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NOTICE

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Review

The sulfolipids*

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[Received for publication October 10, 1960]

The ubiquity of sulfate in biology, mainly bound by ester linkage, is attested to by the large number of classes of such compounds which have been described and are being newly discovered. A sulfate chemistry, perhaps as extensive as we now know for phosphate, is within the realm of possibility. The list of such compounds now includes: (*a*) the sulfated mucopolysaccharides; (*b*) the steroid sulfates; (*c*) the phenol sulfates, including tyrosine-O-sulfate found free or in peptide linkage, triiodothyronine sulfate, and triiodothyroacetic acid sulfate; (*d*) bilirubin sulfate; (*e*) the arylamine sulfates; (*f*) choline sulfate in lichens; fungi and marine algae; and (*g*) the sulfolipids.[1]

Although the history of the study of sulfur associated with lipid dates back to at least the 1880's (1, 2, 3), subsequent investigation in this area has been characterized by sporadic periods of activity. Within the past few years, with the development of methods for the separation and isolation of these compounds from related substances, in large part due to the efforts of Lees *et al.* (4, 5) and Radin *et al.* (6, 7, 8), there has been a renewed interest in their biosynthesis and metabolism. The relative importance of the sulfolipids in biology is implied by the finding that in certain forms the sulfur-containing lipids challenge the phosphatides as the major ionic lipid component. With the advance in our understanding of mechanisms concerned in the formation of the complex sphingolipids (9 to 12) and of the enzymatic reactions leading to the activation and transfer of sulfate to a host of acceptors (13 to 22), the point has been reached where systematic studies on the cell-free biosynthesis of sulfolipids can be attempted.

This review will attempt to summarize what little is known regarding the sulfolipids as concerns their chemistry, procedures used in separation and isolation, and studies on the biosynthesis and metabolism of these compounds *in vivo* and *in vitro*. Since much of this work is of relatively recent origin and remains necessarily incomplete, this review will be more in the nature of a progress report designed to stimulate interest in what appears to be an exciting and promising area.

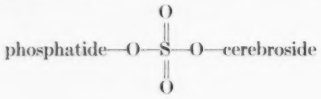
BRAIN SULFATIDE

Chemical Characterization. The sulfur-containing lipid which has been most thoroughly investigated and about which we know most is the so-called sulfatide found in normal mammalian brain. In 1874 Thudichum (1, 2, 3) reported the presence of sulfur in a preparation of cerebrosides prepared from brain. Brain protagon, the white mass resulting after exhaustive acetone-ether extraction of brain, was thought by many to represent a single lipid species with both sulfuric and phosphoric acids in the structure of its molecule. Thudichum, however, believed that the sulfolipid was a distinct lipid, having some properties in common with the phosphatides. Unfortunately he failed to separate the two substances. The ratio of sulfur to phosphorus in his purest samples was 3:2. Some years later Koch (23) isolated a sulfate-containing lipid from an ether-insoluble residue which contained 1.91% sulfur, 1.80% phosphorus, and 12.8% sugar. Koch was led to the conclusion that the sulfolipid contained an equimolar proportion of sulfuric and phos-

* This work was aided by a Faculty Research Associate Award from the American Cancer Society.

[1] Sulfolipid is used here to refer to all lipid-soluble compounds which contain sulfur in covalent linkage. Abbreviations used: UDP-gal = uridine diphosphate galactose; acyl-CoA = acyl-Coenzyme A; ATP = adenosine triphosphate; ADP = adenosine diphosphate; PP = pyrophosphate.

phoric acids. The analytical data led him to formulate the following structure:

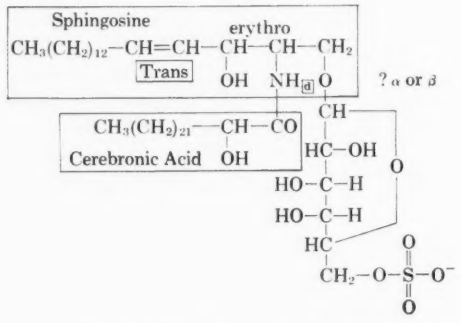


Thus the phosphatide-cerebroside-sulfatide of Koch contained all the elements that were supposed to be parts of protagon. This controversy was finally settled by Levene (24) who, in studies on the lipids of beef brain, isolated a sulfur (2.66%)-containing lipid which was entirely free of phosphorus. Later, in work on the characterization of the haptenic substance present in the protagon of beef brain and horse kidney, Landsteiner and Levene (25, 26) isolated an orcinol-positive lipid, having 2.87% sulfur and no phosphorus. So the matter lay until relatively recently when, as will be discussed later, the issue of a phosphate-containing sulfatide has again been raised.

It was not until 1933, however, that this substance was characterized chemically. At this time Blix (27) was able to isolate from normal human brain a sulfatide, as its potassium salt, free of phosphorus, which amounted to 20% to 25% of the total cerebrosides. Its constituents, upon hydrolysis, were cerebronic (hydroxylignoceric) acid, sphingosine, galactose, and sulfate. The composition suggested a compound made up of one part of each of the above substances. The position of the sulfate group was not determined, but Blix suggested that it was probably esterified with the galactose. The galactose was thought to be glycosidically linked to one of the hydroxyl groups of sphingosine and the fatty acid in amido linkage with the sphingosine moiety.

Little, if any, work had been done on the cerebroside sulfuric acid ester from the time of Blix's isolation and characterization until 1951, when Nakayama (28) examined the question of the position of the sulfate group. From his unsuccessful efforts at tritylation of the cerebroside sulfate, he concluded that the primary hydroxyl group of the galactose was esterified by sulfate. Thannhauser *et al.* (29), however, were also unable to tritylate beef brain cerebrosides under the same and more vigorous conditions, so that the negative evidence of Nakayama did not permit any conclusion as to the structure of the cerebroside sulfate. These investigators, working with relatively pure cerebroside sulfate ester (although obtained in poor yield) isolated from beef brain (30), were able to methylate the compound, and by identification of the products of methanolysis, were able to show unequivocally that the sulfuric acid must be esterified to the primary hydroxyl function on carbon-6 of the galactose of the sulfatide.

Based on the work of Blix (27) and Thannhauser *et al.* (29) and in analogy with the known structure of the cerebrosides and sphingomyelin (31 to 39), it is assumed that the structure for cerebroside sulfate may be written as follows:



Cerebroside Sulfate (Sulfatide)

Recently Jatzkewitz (40) was able to separate chromatographically "sulfatides" with cerebronic, lignoceric, or possibly nervonic acid as the fatty acid component. The strongly metachromatic reaction given by sulfatide with toluidine blue suggests the possibility of a polymerized macromolecule (41).

Solubility and Chemical Properties. The solubility of sulfatide in various solvent systems has been exhaustively studied by Lees *et al.* (4, 5) and has been made the basis of extraction and separation procedures. The divalent cation salts of brain sulfatide are more favorably disposed to the chloroform phase for solution than the sodium or potassium salts (7).

The results of partial acid and alkaline hydrolysis of brain sulfatide have been reported only recently (5). Of interest, and contrary to expectation, based on Klenk's experience with the cerebrosides (42), upon hydrolysis with saturated $Ba(OH)_2$ there was a relatively slow appearance of free amino nitrogen, while the galactosidic linkage of the sulfatide was cleaved relatively easily. Unlike the case with the cerebrosides, such treatment of sulfatide resulted in little or no liberation of psychosine (galactosylsphingosine). It should be noted that the infrared absorption of the sulfuric acid ester grouping has been made the basis for the microdetermination of sulfatide (43).

Separation of Brain Sulfatide. Until recently, the methods used for the isolation and preparation of brain sulfatide have been complicated and tedious, and have resulted in extremely poor yields (27, 30). Within the past few years relatively simple column (Florisil® plus ion exchange) (6, 7, 8, 44) and paper (45, 46) chromatographic techniques have been developed for

the separation of sulfatide from closely related contaminating substances. Lees *et al.* (4, 5) have devised a procedure for obtaining brain sulfatide in good yield based on the distribution of brain lipids between the two phases of a series of related solvents.

?Phosphate-containing Sulfatide. The question of the existence of a phosphate-containing sulfatide, which had been considered resolved with Levene's work (24, 25, 26), has been raised again. Radin *et al.* (6) found that about 0.3% of the brain lipid phosphorus (after removal of the phosphatides by Florisil®) was retained by ion exchange resins. In subsequent work these workers (7) reported that when P^{32}-phosphate was injected into rats and the sulfatides were isolated as the barium salt, about 1.3% of the total lipid activity was found in this fraction. Attempts to separate *in vivo*-labeled P^{32} and S^{35} in the sulfatide fraction were unsuccessful. Similarly, Lees (4) found by the method of "linked distributions" that half of the sulfatides of the starting lipid material could be obtained in two main fractions, and that one fraction was phosphorus-free and the other contained both sulfur and phosphorus.

For the first time, by the development of newer methods for the preparation of sulfatides, these two groups of workers were able to isolate this material in respectable yield. It seemed possible, therefore, that previous workers were able to obtain a phosphorus-free sulfatide fraction simply because that which was intimately associated with phosphorus had been discarded by the relatively crude isolation procedures. It is pertinent in this connection that paper chromatography of purified sulfatide by the method of Jatzkewitz leads to the appearance of two "sulfatide" spots, one of which, according to Jatzkewitz (45), contains phosphate, although not in sufficient amount to be part of the sulfatide molecule (40).

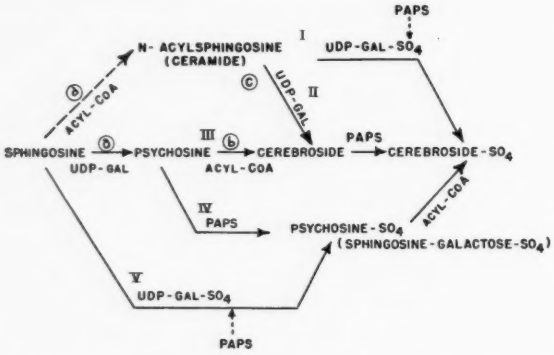
The fallibility of arriving at conclusions concerning covalent linkage based on the similarity of physical properties of substances is not new in lipid chemistry. That solvent solubilities and chromatographic characteristics of lipid compounds may be profoundly altered by trace contaminants of other lipid substances is well known. The recent work of Lees *et al.* (15) suggests strongly that the so-called phosphate-containing sulfatide may be a case in point. These investigators have found the phosphorus-free fraction (I) to be probably pure sulfatide, and the second fraction (II) to be a lipid mixture which could be separated into a phosphatide and sulfatide fraction by passage through a Florisil® column. The phosphatide fraction was composed of a mixture, over half of which comprised phosphatidyl serine; the sulfatide fraction separated by Florisil® was impure, containing cholesterol and other galactose-containing lipids. The ratio of moles of galactose to atoms of sulfur was 1.4:1. Characterization of this fraction remains incomplete, but so far there is no evidence that the sulfatide present in this fraction (II) differs in composition from that in the other fraction (I). The presence of the phosphatide contaminant was shown, however, to alter the chemical reactivity of the sulfatide. Before removal of the phosphatide contaminant by Florisil®, the sulfatide in this fraction (II) is much more resistant to acid hydrolysis than the pure sulfatide of the other fraction (I). After Florisil® treatment, however, the ease with which sulfate is released becomes the same. A similar resistance to acid could be demonstrated with pure sulfatide when phosphatide was added to the hydrolysis mixture.

Tissue Distribution. In addition to the finding of sulfolipid in horse kidney by Landsteiner and Levene (25, 26), there have been other reports of sulfur-containing lipids in tissues other than that of the nervous system. In 1907, Koch (47) found sulfolipids in liver, testes, submaxillary glands, and muscle. Sammartino (48) has reported finding sulfur-containing lipids in the lung. Blix (27) has suggested that the sulfolipid reported to have been isolated from dog and rabbit liver, beef spleen, horse blood, and muscle by Baldi (49), and from the adrenal of cattle and horses by Manasse (50), is the cerebroside sulfuric acid ester. These sulfolipids had not been further characterized until recently, when Green and Robinson (51) were able to demonstrate what appears to be cerebroside sulfate in rat kidney, liver, and spleen, and mouse mastocytoma. As will be noted later, under pathological circumstances sulfatide may accumulate in large amounts in several tissues outside the nervous system.

In Vivo Biosynthesis and Metabolism. One of the earliest studies on the incorporation of radioactive sulfur into the brain was reported in 1945 by Dziewiatkowski (52), who found 0.02% of a dose of orally-administered S^{35}-sodium sulfide to be located in the brain. Boström and Odeblad (53) found the uptake of radioactive sulfate to be highest in the gray matter. The incorporation of parenterally-administered radioactive sulfate into isolated sulfolipid of rat brain has been demonstrated by Holmgård (44). By the administration of C^{14}-galactose and S^{35}-sulfate to rats, Radin *et al.* (7) were able to measure the turnover of chromatographically-isolated brain sulfatide and compare its metabolism with that of cerebroside. It was found that the rate of incorporation of C^{14}-galactose into sulfatide was slower than that into cerebroside. The rates were compatible with the cerebroside's being the precursor

of the sulfatide. No turnover of brain sulfatide was demonstrated. In confirmation of the results of Koch and Koch (54), who had found that brain sulfatides continued to accumulate throughout a rat's life span, these workers showed that an adult rat was still able to incorporate S^{35}-sulfate into brain sulfatide. The interesting possibility of a relation between sulfatide accretion and the aging of nerve cells or the memory processes was pointed out by these authors. The continued accumulation of cerebroside sulfate in the brain of human beings has also been found (55). In addition, sulfatides have been found to make an earlier appearance in development than the cerebrosides (55). Similarly, Green and Robinson (56) have compared the rates of turnover of injected $S^{35}O_4$ that was incorporated into brain mucopolysaccharide and brain sulfolipid. The turnover of the sulfate moiety of cerebroside sulfate was extremely slow compared with that of the mucopolysaccharide. By contrast in rat liver and spleen, and mouse mastocytoma labeling of sulfatide and release of radioactivity was much more rapid than in brain (51). It remains to be shown, however, that this turnover of sulfate is representative of the whole sulfatide molecule and not due simply to sulfate transfer or exchange.

Possible Schemes of Cerebroside Sulfate Biosynthesis. Based on available information regarding mechanisms of synthesis of complex sphingolipids and what has been firmly established as concerns the activation and transfer of sulfate, several possible schemes for the biosynthesis of sulfatide can be formulated:



In all schemes the immediate donor of activated sulfate is taken to be 3′-phosphoadenosine, 5′-phosphosulfate (PAPS) (14, 15, 22). From the work of Bandurski and his collaborators (16, 17, 19) and Robbins and Lipmann (18, 20, 21), it has been established that PAPS is formed enzymatically from inorganic SO_4 and ATP by a two-step reaction. The first reaction catalyzes the displacement of inorganic pyrophosphate from ATP with the formation of adenosine-5′-phosphosulfate (APS). The second reaction is the phosphorylation by ATP of the 3′-hydroxyl group of APS to form PAPS:

$$\text{ATP} + SO_4^{-2} \xrightleftarrows{\text{Enz. A}} \text{APS} + \text{PP}$$

$$\text{APS} + \text{ATP} \xrightarrow{\text{Enz. B}} \text{PAPS} + \text{ADP}$$

$$\text{over-all:}\quad 2\,\text{ATP} + SO_4^{-2} \longrightarrow \text{PAPS} + \text{ADP} + \text{PP}$$

That PAPS is the common metabolic donor of activated sulfate has been repeatedly confirmed in a variety of systems with a wide variety of acceptor compounds. The enzyme systems concerned with the transfer of sulfate from PAPS to the acceptor compound have been termed sulfokinases (22).

In schemes I and II, the ceramide is considered to be the precursor of the more complex sphingolipid, whereas in schemes III, IV, and V, the sphingosine base is the precursor. Cleland and Kennedy (10) have demonstrated reaction *a* and probably *b* in microsomes of rat and guinea pig brain, although some suggestive evidence for reaction *c* could be obtained with the whole homogenates of such tissues. These workers were careful to point out that "it is possible that psychosine in an intermediate in cerebroside synthesis, and ceramides the intermediates in the syntheses of more complex glycolipids." Evidence for reaction *d* has been provided by Zabin (11).

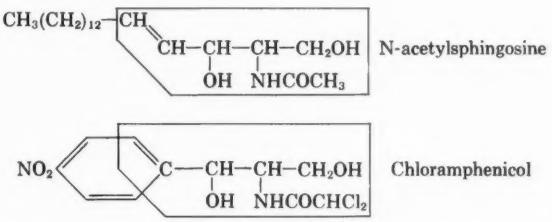
The question of the point in time at which the sulfate is transferred to the hydroxyl function of the sugar moiety has its analogy in mucopolysaccharide biosynthesis. The mechanism of sulfurylation in the formation of sulfated polysaccharides has been the subject of considerable speculation and experimentation (57 to 63). It is possible to conceive of the sulfate's being esterified to the galactose, in the form of a sulfurylated uridine nucleotide (UDP-gal-SO_4), before polymerization occurs in mucopolysaccharide formation or before the sugar is attached to the lipid moiety in the case of the biosynthesis of the sulfatide (schemes I and V). Precedence for such a nucleotide intermediate has been provided by the finding of Strominger (64) of uridinediphospho-N-acetylgalactosamine-4-sulfate in hen oviduct. Although this compound would appear to be an obvious intermediate in chondroitin sulfate synthesis, the evidence to date does not suggest such a role (62). In fact, the direct sulfurylation of the already completed polysaccharide has been shown to take place (61, 62, 63).

In Vitro Biosynthesis and Metabolism. The available data are as yet too incomplete to allow one to select intelligently one scheme over another as the

probable route of sulfatide biosynthesis. Experiments at the cell-free level designed to determine the mechanism of formation of the sulfatide and sulfur-containing lipids in general have been described only recently (65, 66, 67). Evidence has been obtained that extracts of rat kidney and liver as well as brain were able to catalyze the incorporation of radioactive sulfate, $S^{35}O_4$, from $PAPS^{35}$ into a lipid which may be the Blix compound (based on preliminary identification of the brain substance). In fact, kidney and liver preparations were found to be more active in this regard than brain. Despite repeated efforts, no evidence for a compound such as UDP-gal-SO_4 could be obtained, making schemes I and V less likely as pathways of significance in sulfatide synthesis. Of course this possibility is not ruled out. The enzymatic activity resided in a particulate, probably mitochondrial, fraction in young rat brain, whereas, in the liver a high speed (105,000 × *g*) supernatant fraction of the homogenate was active. Interestingly, a similar subcellular distribution of sulfatide in rat brain and liver has been recently reported by Green and Robinson (51). The enzymatic experiments mentioned above are of a preliminary nature and make no distinction between net synthesis and exchange of sulfate in preexisting sulfatide.

Scheme IV is attractive in that all intermediates between the starting sphingosine and final cerebroside sulfate possess considerable water solubility. Efforts to show sulfate-accepting ability for either psychosine or natural cerebrosides, however, have so far not been successful (65). Such experiments, especially in the case of the latter substances, demand cautious interpretation because of unresolved problems of substrate solubility and, hence, availability to the active site of the enzyme. A possible role for the ceramide was indicated by the stimulation of incorporation of $S^{35}O_4$ into sulfolipid by addition of N-acetylsphingosine to the crude brain particulate system.

Sulfurylation of N-acetylsphingosine and Chloramphenicol. A more detailed investigation of the enzymatic systems for sulfate incorporation into lipid from PAPS in the soluble fraction of rat liver has led to the study of an interesting reaction which may be of some importance in sphingolipid metabolism in general. The reactions described above presumably depend on the presence of endogenous lipid substrate precursor as sulfate acceptor. In addition, it was found that the rat liver supernatant fraction was markedly stimulated in its ability to transfer $S^{35}O_4$ from PAPS into lipid when N-acetylsphingosine (the threo isomer was found to be more active than the erythro—whether this is related only to the greater ease of emulsification of the former has not been settled) was added to the incubation mixture. Evidence was obtained with purified protein fractions suggesting the direct transfer of sulfate from PAPS to the primary hydroxyl group of the ceramide to form N-acetylsphingosine-O-sulfate, a compound not previously known. An interesting consequence of these studies was the finding that these same partially purified enzyme fractions were also able to sulfurylate chloramphenicol (the D-erythro isomer is most active), a compound which bears striking structural similarities to the ceramide.



Both possess a 2-amino-1,3-propanediol backbone with two asymmetric centers; chloramphenicol has a benzylic hydroxyl and a dichloracetamido group, whereas in N-acetylsphingosine there is an allylic hydroxyl and an acetamido group. That the same enzyme system was probably involved in the sulfurylation of both compounds was indicated by the ability of the chloramphenicol to compete with the ceramide for sulfurylation in the presence of excess PAPS.

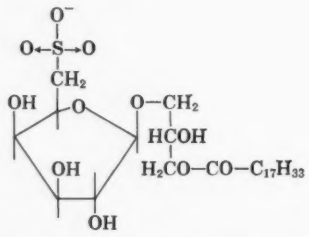
The physiological significance of this reaction is not presently known, but it is of some interest that rat liver, which lacks the ability to sulfurylate choline, is able to sulfurylate the ceramide, whereas *Neurospora*, which can sulfurylate choline, cannot sulfurylate the ceramide. Both systems appear to be absent in rat brain and marine snail extracts (65). Whether the ceramide sulfate is yet another example of a liver detoxification product or has a biological role of its own remains to be determined. The ability of the chloramphenicols to compete with N-acetylsphingosine for sulfurylation warrants a similar examination of other reactions in which the ceramides take part, i.e., sphingomyelin synthesis. Likewise the chloramphenicol free base deserves study as a competitor for the sphingosine base in psychosine formation. Possible implications in sphingolipid disease states are obvious.

Sulfatides in Diffuse Metachromatic Sclerosis. Of considerable interest have been the investigations of Austin (68 to 71) on the nature of the metachromatic straining lipid granules in the urine of children with the so-called metachromatic form of diffuse cerebral sclerosis, a familial progressive demyelinating disease of the nervous system. Pathologically this same meta-

chromatic material is distributed in large excess in many tissues outside the nervous system. This material has been identified by Austin (41, 70, 71), Jatzkewitz (45, 72), and Hagberg *et al.* (46) as sulfatide. This disease appears to constitute a sulfatide lipidosis (71).

SULFOLIPIDS IN PLANTS AND TUBERCLE BACILLI

Recently reports have appeared on the finding of two new sulfur-containing lipids in plants and virulent tubercle bacilli. Benson and his co-workers (73, 74, 75) have described a sulfolipid present in photosynthetic microorganisms and higher plants. Its concentration in *Chlorella* (4×10^{-3}M) exceeds that of the phosphatides. This compound has not been fully characterized, but its properties upon acid hydrolysis suggested to the authors that it might contain a sulfonic acid attached to a galactosyl residue. The proposed structure is as follows:



1-O-(β-6′-deoxy-aldohexopyranosyl 6′-sulfonic acid)-3-O-oleoylglycerol

The other report comes from the laboratory of Middlebrook (76), who has provided evidence that the material responsible for the fixation of neutral red in pathogenic human and bovine varieties of *M. tuberculosis* is a new type of sulfolipid. Characterization is in a preliminary form. There appears to be about 1 mole of neutral red fixed in salt form per atom of sulfur, and an acid equivalent weight of about 3,000. The fraction constitutes 0.1% to 0.2% dry weight of a fully pathogenic human strain. Some evidence has been obtained for there being a group of closely related sulfolipids with slight differences in polarity. The only data on the nature of the sulfur in the lipid come from infrared spectrophotometry, which indicates sulfur-oxygen vibrations.

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Review

Molecular complexes in the isolation and characterization of plasma lipoproteins*

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The formation of insoluble complexes between sulfated polysaccharides and certain plasma lipoproteins was independently described by several investigators in 1955 (1, 2, 3). These complexes are formed at neutral pH and in the absence of organic solvents, and are readily dissociated by increasing ionic strength or by the addition of chelating agents which remove certain metal ions that appear to be necessary for the formation of the complex. Precipitation methods for the isolation and purification of plasma lipoproteins and turbidimetric procedures for their quantitative estimation based on this phenomenon have been developed. These methods satisfy many of the criteria proposed by Lindgren and Nichols (4) for the evaluation of lipoprotein isolation techniques. The mild conditions required for complex formation and dissociation, as well as the convenience and reproducibility of the methods, make them particularly attractive for the investigation of plasma lipoproteins. Comparable isolation procedures involving the formation of complexes between lipoproteins and the neutral polymer polyvinylpyrrolidone have also been described recently (5). In this review the formation of complexes between lipoproteins and macromolecules will be discussed and methods employing complex formation for the isolation and quantitation of plasma lipoproteins evaluated. Since the turbidimetric procedures are comparable in some ways to the older thymol (6) and phenol (7) turbidity tests for the estimation of γ-globulins and lipoproteins, a discussion of these is also included in this review. Fractionation procedures such as the Cohn cold-ethanol Methods 6 and 9 and Method 10, although frequently employed in lipoprotein studies, are not included since they have already been thoroughly discussed in the literature (4, 8 to 16).

Before discussing lipoprotein complex formation, it is appropriate to describe the kinds and properties of the lipoproteins which have been isolated from the plasma of human beings and other animal species in both normal and abnormal states. Plasma lipoprotein fractions represent classes of related, though not identical, molecules. Although these fractions are not homogeneous, their chemical and physical properties and metabolic interrelationships indicate that they do indeed represent distinct classes of related molecules rather than arbitrary fractions defined by the methods used in their isolation. This is most clearly demonstrated by the isolation of the same lipoprotein classes by different fractionation procedures from normal human plasma. However, distinct differences have been found between plasma lipoproteins isolated from normal humans and those isolated from human subjects in certain disease states. Also, the plasma lipoproteins isolated from other animal species are characteristic of the species and may differ considerably from species to species as well as from those in the human. In spite of this, the nomenclature employed to describe human lipoproteins has frequently been indiscriminately applied to animal lipoproteins simply because they correspond by one parameter. The methods which have been developed to separate and characterize human plasma lipoproteins are not generally applicable without modification to other lipoprotein systems. In the sections to follow an attempt is made to correlate the various nomenclature schemes which have resulted from the application of different fractionation procedures to the lipoproteins in normal human plasma. Some of the difficulties involved in applying a classification based on normal plasma to the lipoproteins found in certain disease states are then discussed. Finally, similarities

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and differences between the plasma lipoproteins of various animal species are enumerated to emphasize the problems involved in generalizing fractionation procedures from one species to another.

PLASMA LIPOPROTEIN FRACTIONS ISOLATED FROM NORMAL SUBJECTS

When the flotation rate in an ultracentrifugal field is employed as the defining parameter, the normal human plasma lipoproteins fall into three major categories: the chylomicrons, the low density lipoproteins, and the high density lipoproteins. For reasons to be discussed, the flotation rate, S_f, at density 1.063 has been used most frequently in the characterization of low density lipoproteins. Though perhaps somewhat arbitrary, the choice of this density as the dividing line between the low and high density lipoproteins is corroborated by other parameters of these two groups. Further subdivision of the low density lipoproteins into S_f 0–10 (average density 1.035) and S_f 10–400 (average density 0.98) fractions, though again appearing somewhat arbitrary, also can be justified by other considerations. In this review we shall classify the lipoproteins of normal human plasma as follows: (*a*) chylomicrons; (*b*) S_f 10–400 lipoproteins; (*c*) S_f 0–10 lipoproteins; and (*d*) high density lipoproteins.

Chylomicrons. Chylomicrons were originally defined as the fat particles in chyle or plasma which are visible by dark-field microscopy (17). A precise operational definition based on the isolation of pure chylomicrons has not yet been achieved because of the difficulty in attaining clear-cut separations from the upper S_f spectrum of the low density lipoproteins. Nevertheless, it is felt that true chylomicrons represent an entity distinct from very low density lipoproteins by virtue of their metabolic origin and fate (18). The term chylomicron, in this sense, refers to the vehicle for transport of exogenous triglyceride from the alimentary tract to the blood via the chyle. Chylomicrons are composed mainly of triglycerides, and contain only small amounts of cholesterol, phospholipid, and protein (13, 19 to 24). Part of the protein appears to be similar to, if not identical with, high density lipoprotein (25, 26). Upon entering the blood from the lymph, chylomicrons may acquire additional protein at the expense of high density lipoprotein in the plasma. It is of interest to note here that intravenously infused synthetic fat emulsions similarly associate with high density lipoproteins as demonstrated by ultracentrifugal flotation.[1] Chylomicrons have a density near 0.94 (12) and an S_f greater than 400 (4). Oncley has recently estimated the chylomicron flotation distribution as S_f 10,000 ± 5000 (27). Several flotation procedures at a density of 1.006 have been employed for the isolation of chylomicrons: 9500 × *g* for 10 minutes (28); 9300 × *g* for 30 minutes (18); 26,000 × *g* for 30 minutes (29); and 100,000 × *g* for 30 minutes (23, 25, 29, 30, 31). Some discrepancies in the literature concerning the metabolism of chylomicrons are probably attributable to differences in isolation technique. For example, Dole *et al.* (31) isolated postprandial chylomicrons by centrifugation at 100,000 × *g* for 30 minutes and concluded that dietary fats had little influence on their fatty acid composition. Bragdon and Karmen (29), on the other hand, found that chylomicrons isolated by centrifugation at 26,000 × *g* for 30 minutes did reflect the fatty acid composition of the dietary fat. The chylomicron fraction isolated at 100,000 × *g* probably contained appreciable amounts of S_f 10–400 lipoproteins.

Postprandial lactescence in the serum of normal individuals is caused by chylomicrons (17, 23); however, the assumption that all lactescence in the serum of hyperlipemic subjects is caused by these particles is probably not valid (18, 32). It has been reported that lymph chylomicrons migrate with the mobility of albumin in free electrophoresis (20, 32), whereas plasma chylomicrons have the electrophoretic mobility of α_2-globulins (19, 32, 33, 34). The turbid or lactescent region of the electrophoretic pattern of alimentary lipemic serum differs from that seen in essential, diabetic, or nephrotic hyperlipemic sera. In the former the turbidity is confined to the α_2 region, whereas in the latter it extends across the α_2 and β regions (32). While zone electrophoresis on paper has proved unsatisfactory for the study of chylomicrons because of adsorption, some interesting results have been obtained using starch blocks (34) or columns. Employing the latter, Carlson and Olhagen (35) observed the migration of chylomicrons, as detected by turbidity, from the serum of a patient with essential hyperlipemia as two distinct fractions, one an α- and the other a β-globulin. On the other hand, Kunkel and Trautman (34), employing the starch block technique, observed almost all of the visible lipemia in the α_2 region of normal postprandial serum. These results certainly suggest that more than one type of lipoprotein molecule can contribute to visible lipemia, but the significance of these variants remains to be elucidated. Another method which has been employed to isolate chylomicrons is flocculation with toluidine blue (19) or protamine (20); however, Laurell (33) has recently shown that other lipoproteins are precipitated together

[1] F. A. Kruger, D. G. Cornwell, G. J. Hamwi, and J. B. Brown, unpublished observations.

with chylomicrons by protamine in the presence of fatty acid anions.

S_f 10–400 Lipoproteins. These lipoproteins are defined as molecules having flotation rates between 10 and 400 in a medium of density 1.063. They are generally isolated in the preparative ultracentrifuge by flotation at 100,000 × *g* for 18 to 24 hours in a medium of density 1.006 or 1.019 after preliminary removal of the chylomicron fraction. The fraction isolated under these conditions has been variously designated S_f 10–400 (18, 22, 36, 37, 38), *S*, 12–400 (4, 39, 40, 41), S_f 20–400 (4, 41), and more recently, S_f 16–400 (42). Oncley *et al.* (37) originally suggested that this lipoprotein was concentrated in the S_f 10–100 region with a maximum near S_f 35, and had a mean density of 0.98. In a later paper Oncley (27) suggested that the fraction consisted of two subfractions: S_f 100 ± 60 with a density of 0.958, and S_f 30 ± 8 with a density of 0.990. Lipoproteins with a density near 1.02 and a flotation distribution approximated by S_f 10–17 (22, 38), S_f 5–15 (37), S_f 12–20 (4, 41), or S_f 10 ± 3 (27) are found in normal plasma at a very low concentration. This accounts for the somewhat arbitrary choice by different investigators of the S_f value separating the two main low density lipoprotein groups. It remains to be established whether the observation of lipoproteins in the S_f 10 region represents incomplete separation between these two groups, or actually represents part of a continuum embracing both groups.

The S_f 10–400 lipoprotein fraction contains 49% to 52% triglyceride, 18% to 19% phospholipid, 6% to 7% free cholesterol, 14% to 16% esterified cholesterol, and 7% to 10% protein (22, 37, 43). The cholesterol to phospholipid ratio varies from 0.78 to 0.90 (18, 22, 37, 38, 40). The fatty acid (44, 45), phospholipid (46, 47), and amino acid (48, 49, 50) compositions of the S_f 10–400 lipoproteins have been investigated. These lipoproteins have been reported to migrate as α_2-globulins by starch block electrophoresis (34); however, in view of the results which have been reported for chylomicrons, it would appear that this should be investigated further.

Both S_f 0–10 and S_f 10–400 lipoproteins are found in fraction I + III of cold-ethanol Method 10 (9, 12, 15, 16). Metabolically the S_f 10–400 lipoproteins represent the main vehicle for endogenous triglyceride transport (18, 36). Newly absorbed triglycerides are transported secondarily by them (36). They are synthesized in the liver (51, 52), and converted to S_f 0–10 lipoproteins (53) with the concomitant removal of triglyceride, presumably in the reticuloendothelial system (18, 36, 54, 55). Immunochemical studies indicate that the protein moieties of the S_f 10–400 and S_f 0–10 lipoproteins are closely related, if not identical (56, 57).

S_f 0–10 Lipoproteins. These low density lipoproteins, defined as having an ultracentrifugal flotation rate in the range from 0 to 10 in a medium of density 1.063, are usually isolated by centrifugation at 100,000 × *g* for 18 to 24 hours in a medium of this density (18, 22, 36, 37, 41 to 49, 53, 57, 58, 59). The S_f 10–400 lipoproteins are separated by a preliminary or final centrifugation at density 1.006 or 1.019. Oncley *et al.* (37) isolated and characterized three arbitrary subfractions within the S_f 0–10 spectrum and found a small increase in protein content with decreasing flotation rate. The S_f 0–10 lipoproteins are concentrated in the S_f 3–9 region (37), with a peak at S_f 6 ± 2 (27). These molecules have a mean density of 1.032 (27). Different investigators have used several S_f distributions to describe this lipoprotein fraction: S_f 0–10 (22, 38), S_f 3–9 (18, 36, 37, 57), S_f 0–12 (4, 39, 40, 41), S_f 0–20 (4, 41, 59), and S_f 0–16 (42).

These lipoproteins have the electrophoretic mobility of a β-globulin in moving boundary and zone electrophoresis (34, 37). They are found in fraction III-O of cold-ethanol Methods 6 and 9 (58), and fraction I + III of cold-ethanol Method 10 (9, 12, 15, 16) along with the S_f 10–400 lipoproteins. They differ markedly from the latter in chemical composition containing 9.0% to 12.5% triglyceride, 19% to 32% phospholipid, 7.5% to 9.5% free cholesterol, 37.0% to 39.4% esterified cholesterol, and 21.9% protein (22, 37, 43). The cholesterol to phospholipid ratio varies from 1.30 to 1.45 (18, 22, 37, 38, 40). The protein moiety has the same immunochemical specificity (56, 57) and amino acid composition (48, 49, 50) as that of the S_f 10–400 lipoprotein fraction.

The S_f 0–10 lipoproteins may arise either as the products of S_f 10–400 lipoprotein metabolism (53) or directly from the liver (18, 36, 51, 52). Although they constitute the largest lipoprotein group, their metabolic role, except as the end products of S_f 10–400 lipoprotein metabolism, remains obscure.

High Density Lipoproteins. The high density lipoproteins may be distinguished operationally from the low density lipoproteins by virtue of the fact that they sediment along with other plasma proteins at density 1.063. They may be isolated from the remaining plasma proteins by ultracentrifugal flotation at 100,000 × *g* for 22 hours in a medium of density 1.21 (4, 8 to 14, 22, 34, 41, 43 to 50, 59, 60). Some investigators (4, 12, 41, 59) have distinguished between two high density lipoprotein classes with mean densities of 1.09 and 1.14. Although these two fractions differ in chemical composition (61), they may not represent more than arbitrary fractions of a continuum. Oncley (27) has

recently suggested that only a single high density lipoprotein class exists with a mean density of 1.12. We shall consider these lipoproteins as a single class in this review. These lipoproteins differ from the low density lipoproteins in their amino acid (48, 49, 50) and phospholipid (46, 47) composition as well as immunochemical specificity (57, 62, 63). They contain 4% to 8% triglyceride, 20% to 21% phospholipid, 2% to 3% free cholesterol, 13% to 15% esterified cholesterol, and 46% to 58% protein (22, 43, 61). Cholesterol to phospholipid ratios between 0.38 and 0.58 have been reported (18, 22, 38, 40, 60, 64). The high density lipoproteins exhibit the electrophoretic mobility of α_1-globulins (4, 8 to 14, 34), and are found in fraction IV + V + VI of cold-ethanol Method 10 (9, 12, 15, 16). They have also been separated from low density lipoproteins by paper chromatography (65).

As mentioned in the section on chylomicrons, the latter appear to contain a protein constituent similar in many ways to high density lipoprotein. It has been suggested that this lipoprotein may be liberated as such during the metabolism of chylomicrons in the reticuloendothelial system (36). High density lipoproteins have been shown to be synthesized in the liver (66). At least part of the protein of chylomicrons appears to be synthesized in the intestinal wall (23). The relation of this synthesis to the high density lipoproteins is not known.

A lipoprotein with a density greater than 1.21 has been isolated and partially characterized (22, 40, 61, 67). It contains only a small part of the total plasma phospholipid but is rich in lysolecithin (67). A more thorough investigation of this lipoprotein is necessary before the methods for its isolation and its significance in lipoprotein metabolism can be assessed.

The isolation and characterization of plasma lipoproteins is complicated by the rapid equilibration or exchange of lipid components including cholesterol (68, 69), phospholipid (70, 71, 72), and tocopherol (73) between the different lipoprotein fractions. Exchange between the triglyceride (36) or protein (53, 74) moieties of different lipoprotein fractions has not been demonstrated; however, a very rapid equilibration between the lipid-free protein moiety of the high density lipoprotein fraction and other lipoproteins has been reported (60).

PLASMA LIPOPROTEIN FRACTIONS ISOLATED FROM HUMAN SUBJECTS IN DISEASE STATES

Many disease states are accompanied by marked alterations in the plasma lipoproteins. These alterations may involve the elevation or diminution of normal lipoprotein fractions, changes in the S_f distribution within a lipoprotein fraction, or the appearance of atypical lipoproteins. Methods established for the fractionation of lipoproteins in normal human plasma can be applied in disease states where a normal fraction is elevated or depressed. This is illustrated in the investigation of plasma from hypercholesterolemic subjects. While the S_f 0–10 lipoprotein fraction is markedly elevated (4, 10, 18, 40, 59, 75, 76), it is characterized by normal cholesterol to phospholipid and fatty acid ester to cholesterol ratios (18, 76). The elevated S_f 0–10 fraction does not affect the isolation and characterization of other lipoprotein fractions (18, 76). The concentration of the high density lipoproteins is diminished in hyperlipemia and biliary cirrhosis (18, 38, 40, 76, 77). Fractionation at a density of 1.063 indicated that these lipoproteins had a low cholesterol to phospholipid ratio (18, 40). Baxter *et al.* (38), in a study of hyperlipemic sera from nephrotic individuals, found, however, that the cholesterol to phospholipid ratio of the high density lipoprotein fraction was normal when lipoproteins with a density greater than 1.21 were not included in the fraction.

It is more difficult to apply standard isolation procedures to plasma from hyperlipemic subjects. The S_f 10–400 lipoprotein fraction isolated from these is lactescent and contains molecules rich in triglyceride (18, 38), and with an elevated flotation rate (4, 53, 75). A large lipoprotein fraction is frequently isolated from lactescent sera by ultracentrifugal flotation at 9300 $\times$ g for 30 minutes (18). This procedure isolates postprandial chylomicrons from normal plasma; however, the composition of the "chylomicron fraction" from normal postprandial plasma is different than that of the "chylomicron fraction" from the plasma of hyperlipemic individuals (18). Jobst and Schettler (78) isolated chylomicrons by ultracentrifugal flotation at 22,000 $\times$ g for 60 minutes and found significant differences in composition between the "chylomicron fraction" from the serum of individuals with essential hyperlipemia as compared to alimentary chylomicrons. The "chylomicron fraction" from subjects with essential hyperlipemia contained less triglyceride and was generally similar in composition to S_f 10–400 lipoproteins (18, 78). Thus ultracentrifugal flotation may not distinguish between chylomicrons of alimentary origin and large low density lipoproteins with an enhanced flotation rate.

Elevated S_f 10–400 lipoprotein fractions isolated from hyperlipemic individuals do not represent a simple proportional elevation of lipoproteins normally present

in this part of the S_f spectrum. They show a wide variation in chemical composition (18, 38, 40, 76). Cholesterol to phospholipid and fatty acid ester to cholesterol ratios may be increased or decreased from the normal range (18, 38, 76). In nephrosis the cholesterol to phospholipid ratio increases with increasing lactescence (38); this ratio is sometimes diminished in the corresponding lipoprotein fraction isolated from markedly lactescent sera from individuals with idiopathic hyperlipemia (18). An elevated S_f 10–20 lipoprotein, intermediate in composition and properties between the S_f 0–10 and S_f 10–400 lipoproteins of normal serum, has been found in some hyperlipemic sera (18, 38, 75, 76). Thus different hyperlipemias may involve elevations in specific regions of the S_f 10–400 lipoprotein spectrum, extension of the range beyond S_f 400 (75), or perhaps even the appearance of atypical lipoproteins not normally present in the low density lipoprotein spectrum.

S_f 0–10 lipoprotein fractions isolated from the sera of hyperlipemic subjects also differ from those isolated from normal sera. Cholesterol to phospholipid ratios are lower than normal, while fatty acid ester to cholesterol ratios are increased (18, 38, 76). These alterations in composition are most significant when the S_f 0–10 lipoprotein concentration is very low, as it is in markedly lipemic sera (18, 38, 76). These lipoproteins may represent a small residual fraction remaining after the disappearance of the major S_f 0–10 fraction, or they may represent atypical molecules synthesized in the hyperlipemic state.

The isolation and characterization of the lipoproteins from the sera of subjects with biliary cirrhosis present special difficulties. Whereas high density lipoproteins are greatly diminished or even absent (40, 77, 79), low density lipoproteins within the S_f 0–20 range are greatly increased (40, 75, 79, 80). However, these low density lipoproteins are atypical. Although they migrate electrophoretically as β-lipoproteins (77), their cholesterol to phospholipid ratio is more like that of the high density lipoproteins (77). Russ *et al.* (77) subjected the plasma of a biliary cirrhotic to cold-ethanol Method 10 and found β-lipoproteins in fraction I + III, IV + V, and VI. Normally, fraction VI contains only traces of lipid; in this case it represented the largest fraction. Of the three fractions, only I + III reacted immunochemically with antisera to normal low density lipoprotein. The unfractionated plasma, upon analytical ultracentrifugation, revealed three lipoprotein species: S_f 13 representing two-thirds of the total, and S_f 10 and S_f 7 equally representing the remainder.

It is thus evident that even within the human species the characterization of lipoprotein classes by a single parameter is inadequate and may lead to erroneous identification, especially in abnormal states. The relationships established between the various parameters of the lipoproteins in normal human plasma do not necessarily hold in abnormal conditions.

PLASMA LIPOPROTEIN FRACTIONS ISOLATED FROM OTHER ANIMAL SPECIES

Pedersen (81) first demonstrated the existence of the low density lipoproteins in human plasma by ultracentrifugation, which he designated by the term "X-protein," and also noted that a comparable protein was absent from other animal plasmas. This finding was substantiated by the use of cold-ethanol fractionation procedures (82). When applied to human plasma, a major portion of the lipoproteins appeared in fraction II + III; these were identified as β- or low density lipoproteins. By contrast, comparable fractions from animal plasmas contained very little lipoprotein (82). Most of the lipoproteins of animal sera appear in fractions IV-1 and IV-2, and are α-lipoproteins by electrophoresis (82). Both analytical and preparative ultracentrifugation have demonstrated that many animal species contain large amounts of high density and only small amounts of low density lipoproteins (40, 83). The correlation between low density, β, and fraction II + III lipoproteins on the one hand, and high density, α, and fraction IV lipoproteins on the other, which has been observed in human plasma, has not been established generally for the plasma of other animals. Only in the case of dog plasma have the lipoproteins been isolated and characterized by a variety of methods. Lipoprotein fractions obtained from human and dog plasma by cold-ethanol Method 10 (84), paper electrophoresis (85, 86), ultracentrifugal flotation (40), and analytical ultracentrifugation (83) are compared in Table 1. The agreement of the distribution of dog plasma lipids between the low density lipoproteins (fraction I + III and those with density 1.019–1.063) and the high density lipoproteins (fraction IV + V + VI and those with density >1.063) by procedures dependent upon solubility and ultracentrifugal properties would seem to indicate that the dog lipoprotein fractions correspond with the human. The paper electrophoretic data indicate a larger proportion of β-lipoprotein in the dog plasma than is indicated by the ultracentrifugal and solubility data. However, it may be noted that the dog serum cholesterol levels reported here are somewhat higher than usual (less than 150 mg/100 ml). In a paper electrophoretic study of dog serum by Fasoli *et al.* (87), the slow-mi-

grating fractions accounted for only about 15% of the total cholesterol.

Although there appear to be two types of lipoproteins in dog plasma as demonstrated by three independent criteria, it is of interest to note that whereas the lipid compositions of comparable fractions isolated from human plasma differ widely, those isolated from the dog are quite similar at least with regard to the cholesterol to phospholipid ratio. Further chemical characterization with regard to other constituents such as triglyceride and the protein moiety is necessary before the significance of these fractions can be evaluated. This has been attempted in a study of N-terminal amino acids, which indicated that dog lipoproteins in the density 1.019–1.063 and density 1.063–1.20 fractions had different amino acid residues (25). Analytical ultracentrifugation (Table 1) demonstrates further differences between dog and human low density lipoproteins. In dog plasma these exhibit flotation rates, S_f 1–3 and $-S$ 23, which correspond to a minor human lipoprotein fraction, HDL_1, not separated from the major low density lipoproteins by ultracentrifugal flotation at density 1.063 (59). When dog lipoproteins are prestained with Sudan black B and separated by ultra-

TABLE 1. Isolation and Characterization of Human and Dog Plasma Lipoproteins

Method and Fraction	Human			Dog		
	Cholesterol	Phospholipid	Cholesterol / Phospholipid	Cholesterol	Phospholipid	Cholesterol / Phospholipid
Cohn Method 10 (84)		*mg/100 ml*			*mg/100 ml*	
Plasma	189	225	0.84	134	257	0.53
Fraction I + III*	123	98	1.25	10.2	23.7	0.61
Fraction IV + V + VI*	61	117	0.52	114	234	0.50
Paper Electrophoresis (85,† 86‡)						
Serum	245	259	0.94	243	455	0.53
α_2	27.5	36.0	0.77			
$\beta + \gamma$§	158	128	1.24	61	127	0.48
α_1	59.5	95.0	0.63	182	328	0.55
Ultracentrifugal Flotation (40)						
Serum	179	226	0.79	150	362	0.41
d <1.019	23	28	0.82	2	10	0.20
d 1.019–1.063	103	74	1.39	10	19	0.53
d >1.063	49	123	0.40	127	325	0.39

Analytical Ultracentrifugation (83)	Lipoprotein	
	mg/100 ml	*mg/100 ml*
Density 1.06		
S_f 20–60	20	0#
S_f 10–20	28	0
S_f 9	0	0
S_f 3–8	200	0
S_f 1–3	0	38
Density 1.21		
$-S$ 40–70	30	0#
$-S$ 35	0	0
$-S$ 30	200	0
$-S$ 23	14	77
$-S$ 6	0	0
$-S$ 3	140	400

* Fractions I + III and IV + V + VI are called fractions C + D and A in this study of dog serum (84).
† Paper electrophoresis of human serum (85).
‡ Paper electrophoresis of dog serum (86).
§ Fraction $\beta + \gamma$ signifies β-lipoproteins and lipoproteins trailing from the origin (85, 86).
# Different dogs were used in these ultracentrifugation experiments (83).

centrifugal flotation in a density gradient,[2] a lipoprotein continuum is found instead of the separation into discrete low and high density fractions observed with human serum (88, 89).

As in the human, thyroid deficiency in dogs leads to alterations in the plasma lipoprotein pattern. These changes are quite different, however. In the human there is a marked increment in the S_f 0–10 lipoproteins, a small increment in the S_f 10–400 lipoproteins, and essentially no change in the high density lipoproteins (18). Milch *et al.* (90), studying I^{131}-thyroidectomized dogs, observed marked elevations in high density lipoproteins as well as in the S_f 0–12, S_f 12–20, and S_f 20–400 lipoproteins. In addition, they noted an almost twofold increase in the cholesterol to phospholipid ratio of the total serum lipids. This would indicate a change in the nature of at least some of the lipoproteins since, as indicated in Table 1, none of the lipoproteins isolated from normal dog plasma exhibit cholesterol to phospholipid ratios this high. The lipoproteins of thyroid-deficient dogs fed cholesterol have also been studied, and similar elevations in the various lipoprotein fractions found by cold-ethanol Method 10 (91) and electrophoresis (92). The cholesterol to phospholipid ratios of the total serum lipids (91, 92), as well as of both the high and low density fractions (91), were markedly elevated. An investigation of N-terminal amino acids in the lipoprotein fractions of hypercholesterolemic dogs, similar to the recent study by Shore and Shore (93) on hypercholesterolemic rabbits, would be highly significant.

In some respects the study of lipoprotein metabolism in animals like the dog is less complicated than it is in the human. For example, chylomicron metabolism can be followed in the dog with little chance of interference by low density lipoproteins in fractionation procedures (31, 94, 95). The guinea pig may offer certain advantages in the study of low density lipoproteins since there is a virtual absence of high density lipoproteins in guinea pig plasma (83). However, as has been pointed out, plasma lipoproteins differ from species to species in their physical and chemical properties, and perhaps also in their metabolic behavior. It should also always be kept in mind that lipoprotein fractionation procedures may require modification before they are applied to the plasma of a given species.

THE NATURE OF THE INTERACTIONS OF PLASMA LIPOPROTEINS WITH OTHER SUBSTANCES

The complexes formed by the interaction of plasma lipoproteins with certain sulfated polysaccharides bear at least a formal resemblance to those complexes isolated from biological systems which contain a protein bound to one of a variety of polyanions such as heteropolysaccharides, nucleic acids, or phosphoproteins. Polysaccharide anions such as hyaluronic acid in synovial fluid and chondroitin sulfate in cartilage form complexes with proteins in which electrostatic attractions between cationic groups on the protein and anionic (sulfate and carboxyl) groups probably play a major stabilizing role since the complexes are broken by the addition of alkali or increasing the ionic strength of the medium (96). It has been suggested that the anionic groups of mucopolysaccharides in connective tissue are in ion exchange equilibrium with metal ions and the cationic groups of the protein (97, 98). In a study of the various factors involved in the binding of protein to mucopolysaccharide in connective tissue, Loeven (99) concluded that although charge considerations are of primary importance, structural factors may alter the degree of binding. Partridge and Davis (100), studying chondroitin sulfate complexes in cartilage, arrived at similar conclusions, attributing the more specific aspects of the binding to hydrogen bonding and other short-range forces.

Nucleoproteins containing nucleic acid polyanions and either protamines or histones are undoubtedly stabilized mainly by electrostatic forces since the complexes are readily disrupted by increasing either the pH or the ionic strength of the medium (101, 102, 103). The situation is somewhat more complicated when the interaction between nucleic acids and other proteins is considered (101). High ionic strength alone does not suffice to disrupt these complexes. However, the components can be caused to separate by the use of protein denaturants such as urea, guanidine hydrochloride, or sodium dodecylsulfate. Conversely, the interaction between a given globular protein and nucleate polyanion to form a complex is generally weaker after denaturation of the protein than before denaturation. This is true even after the denaturing agent is removed, and also for heat denatured proteins. It therefore appears that certain structural features of the native protein in addition to net charge, which remains essentially unchanged with denaturation, contribute to the binding of globular proteins to nucleic acid polyanions. Recent studies on the formation of soluble and insoluble complexes between spermine and soluble ribonucleic acid (104) and spermine and synthetic polynucleotides (105) show that polynucleotide structure is also important in determining the type of complex formed.

Molecular complexes between proteins and phosphoprotein polyanions have not been studied as thor-

[2] D. G. Cornwell and F. A. Kruger, unpublished observations.

oughly as the heteropolysaccharide and nucleic acid complexes; however, a phosphoprotein complex obtained during the fractionation of hen egg yolk has been partially characterized. Yolk, diluted with 10% sodium chloride, exhibits two sedimenting fractions in the ultracentrifuge (106). Although the major sedimenting fraction, isolated and dissolved in 10% sodium chloride, is homogeneous by sedimentation and electrophoresis, it has been shown to contain three proteins: lipovitellin (lipoprotein), phosvitin (phosphoprotein), and γ-livetin (globulin) (107). The γ-livetin component may be separated by sedimentation or electrophoresis in alkaline solution (pH 9) at low ionic strength ($\mu = 0.3$) or may be partially purified by precipitation from a 40% saturated ammonium sulfate solution (107, 108). The molecular complex is also disrupted in 0.4 M magnesium sulfate and phosvitin precipitated from this medium as a magnesium complex (109, 110). Dissociation in alkali or in magnesium sulfate both indicate that electrostatic bonding is important in stabilizing this complex system isolated from egg yolk. Its stability in 10% sodium chloride suggests that other factors assist in holding the molecular complex together. The structure of naturally occurring molecular complexes between phosphoproteins and lipoproteins in the whole yolk is more difficult to ascertain. The molecular complex containing lipovitellin, phosvitin, and γ-livetin may be an isolation artifact. Schjeide and Urist (111) have isolated granules or giant yolk complexes from egg yolk which contain only phosphoprotein (phosvitin) and dense and light lipoproteins (lipovitellin and lipovitellenin, respectively). Livetins were found only in the yolk fluid. Calcium ions are bound to the naturally occurring phosphoprotein, and the removal of these ions during isolation liberates anionic groups (112). These groups may then form electrostatic bonds with the cationic groups of a livetin and form the complex described by Joubert and Cook (107).

Other examples of complex formation between oppositely charged macromolecules include the association of protamine and heparin (113), the formation of a heparin-albumin precipitate below the isoelectric point of albumin (114, 115), the formation of an albumin-polylysine precipitate above the isoelectric point of albumin (116), and the coprecipitation of γ-globulins and low density lipoproteins in their interisoelectric region (117). Protein-protein and protein-synthetic polyelectrolyte interactions have been reviewed by Waugh (118) and Sela and Katchalski (119). Since these complexes are readily disrupted by altering the pH or increasing the ionic strength, it is difficult to establish the contribution of binding forces other than electrostatic forces. Furthermore, their formation is relatively nonspecific and does not appear to require unique structural relationships. Phytic acid, for example, precipitates most extracellular proteins in the region between pH 2 and pH 4 (120).

Although it was originally believed that heparin formed complexes with plasma proteins only in the pH region where the proteins were positively charged (121), evidence was obtained by Jaques (122), from considerations of solubility behavior, that complexes existed between heparin and proteins even under conditions where both macromolecular species carried a negative charge. A number of investigators have subsequently demonstrated that heparin forms molecular complexes with both albumin and low density lipoproteins in serum at hydrogen ion concentrations where the proteins carry a net negative charge (114, 115, 123 to 126). Complexes between fibrinogen and sulfated polysaccharides have been described in which protein and polyanion specificity at a pH above the isoelectric point would appear to indicate that polymer configuration and other secondary valence forces as well as electrostatic bonds contribute to the formation and stability of the complexes (127 to 130). Complex formation as determined by either electrophoretic behavior or precipitation is nevertheless very sensitive to changes in pH and ionic strength, generally decreasing with increases in either. However, Smith and Von Korff (129) have described the "salting-out" precipitation of fibrinogen-heparin complexes from concentrated sodium chloride solutions as well as the "salting-in" effect found at lower ionic strengths. Other evidence for nonelectrostatic interactions between proteins and polysaccharides has been obtained from studies on complex formation between glycogen and concanavalin-A (131, 132), between albumin and dextran (133), and between fibrinogen and dextran (134, 135). Glycogen which has been methylated no longer forms complexes with concanavalin-A (132). Cifonelli *et al.* (136) observed that whereas heparin precipitated concanavalin-A more efficiently than glycogen, no precipitation occurred with chondroitin sulfate or hyaluronic acid under similar conditions. These observations indicate that polymer structure and hydrogen bonding are important factors in complex formation. It is of interest to note here that Peterson and Sober (137) implicate nonionic bonding forces as contributing to departures from electrophoretic order in the elution of proteins from polyelectrolyte cellulosic absorbants. The neutral polymers polyvinylpyrrolidone[3] and poly-

[3] PVP. Technical Bulletin, Antara Chemicals, New York 14, N. Y.

N-vinyl-5-methyl-2-oxazolidinone[4] have recently been shown to exhibit rather unique and specific complexing capacities for certain organic compounds as well as certain proteins. Interactions with the latter are discussed more completely later in this review.

The complexes formed by the interactions between polyanions and lipoproteins are comparable in many ways to the colloid complexes described by Bungenberg de Jong (138). We have already considered the interaction of polyanions with proteins below their isoelectric point; these result in the formation of dicomplex systems. In many instances, however, the presence of certain metal ions is essential for the formation of a complex. Bungenberg de Jong refers to these as tricomplex systems, amphion-cation-anion, in which the amphion may be a protein or other ampholyte such as egg lecithin, the cation a di- or trivalent metal ion, and the anion either a polyanion or a micro anion. Typical examples are: egg lecithin-La(III)-arabinate; isoelectric gelatin-Zn(II)-chondroitin sulfate; isoelectric gelatin-Cd(II)-thiocyanate. The role of metal ions in the formation of lipoprotein-polyanion complexes is discussed in the section on lipoprotein-polysaccharide complexes.

Albumin and the low density lipoproteins form complexes with simple anions above their isoelectric points. Albumin-anion interactions have been studied by a number of investigators and are discussed in reviews by Klotz (139) and Foster (140). The literature on low density lipoprotein-anion interactions is less extensive, but several important studies have appeared on the binding of free fatty acids (141, 142) and methyl orange (143). Goodman and Shafrir (142) showed that both S_f 10–400 and S_f 0–10 lipoprotein fractions bind a small number of fatty acid anions at strong binding sites, and a large number at weaker binding sites. While binding affinities are somewhat greater with the S_f 10–400 lipoproteins, they both bind the same number of fatty acid anions (142). These interactions may be compared with the binding of fatty acid anions by plasma albumin studied under the same conditions (144). Albumin binds more fatty acid anions at two somewhat stronger classes of binding sites than do the low density lipoproteins. However, more fatty acid anions are bound more strongly at the weaker binding sites of the low density lipoproteins than at the weakest class of binding sites of albumin. Rosenberg *et al.* (143) found that methyl orange binding corresponded rather closely in low density lipoprotein and albumin systems. These similarities in anion binding would appear to indicate that similar mechanisms are involved in anion interactions with albumin and the low density lipoproteins. Foster (140) has summarized the results of many studies on the binding by albumin of dyes and detergents and concluded that the tendency for water molecules to form a maximum number of hydrogen bonds with each other is a major factor in promoting protein-anion interactions. Aggregation of anions into micelles and the association of anions with proteins permit increased hydrogen bonding between solvent molecules, since hydrophobic groups constitute discontinuities in the surrounding hydrogen-bonded aqueous environment. These discontinuities may be minimized by the association of protein and anion hydrophobic groups (145). Foster (140) further suggests that albumin may assume a structural configuration in which hydrophobic groups are oriented toward the aqueous phase and are therefore available for nonionic bonding. Goodman and Shafrir (142) state that the large number of weaker binding sites on the lipoprotein molecule may result from hydrophobic interactions. The relative availability of hydrophobic groups may be characteristic of both albumin and low density lipoprotein molecules, and this structural feature influences the formation and specificity of protein-anion and protein-polyanion complexes.

The interaction of phenols with proteins has been attributed to the formation of hydrogen bonds between the phenolic hydroxyl groups and the peptide linkages in proteins (145 to 148). Mejbaum-Katzenellenbogen (149) studied the interaction of several proteins with naturally occurring phenolic polymers, the tannins, and found that insoluble complexes were formed at or below the isoelectric points of the proteins. These complexes did not dissociate at high ionic strength; however, they were dissociated by the addition of caffeine (150). Hydrogen bonding between phenols and caffeine may explain this phenomenon. The interaction of monomeric phenols, thymol and phenol, with proteins is somewhat more specific and will be discussed in the next section. It would appear, however, that considerations similar to those discussed above with reference to albumin and lipoprotein interactions with fatty acid and other anions may also apply to the interactions of proteins with phenols.

THE TURBIDIMETRIC ESTIMATION OF PLASMA LIPOPROTEINS WITH PHENOLS

In 1944, Maclagan (6) described a thymol turbidity test for the estimation of serum globulins in liver disease. He analyzed the precipitate formed on adding buffered aqueous thymol to human serum and found that it contained protein nitrogen, lipid phosphorus, and cholesterol as well as thymol. Cholesterol to phospholipid ratios

[4] Molecular Complexing with Devlex® Resins. Technical Bulletin, Dow Chemical Company, Midland, Mich.

calculated from these data (average 1.43) indicate that the precipitate contained low density lipoproteins. Kunkel and Hoagland (151) found that thymol reacted with both γ-globulins and serum lipids. They also noted that the turbidity resulting from the γ-globulin did not occur at elevated ionic strengths, whereas that due to the serum lipids was unaffected by increased ionic strength. However, prior extraction of lipids from hepatitis serum with ether prevented the production of turbidity with γ-globulins. Reintroduction of serum lipid in the form of serum giving little thymol turbidity itself restored the turbidity (151, 152). It thus appears that the presence of serum lipoproteins is essential in the thymol turbidity reaction. Electrophoretic studies also indicate that γ-globulins are necessary in the production of thymol turbidity (151, 153). Albumin has an inhibitory effect on the production of turbidity (6, 154); however, this observation has been questioned by Albertsen *et al.* (155), who studied the effect of several purified protein fractions on turbidity. Thymol turbidity seems to depend on complex interactions between thymol, γ-globulins, low density lipoproteins, and perhaps albumin. Nevertheless, several investigators have used thymol turbidity (156) to estimate serum lipids and lipoproteins, especially the neutral fat and chylomicron concentrations (157 to 161). Walther (162) found that thymol turbidity was useful in following serum lipid alterations in a given individual, but it was difficult to correlate with the serum lipid levels of different individuals. The thymol test has been discussed in several reviews (163, 164).

Maclagan (6) investigated a number of phenolic compounds in his study of liver function tests. Kunkel *et al.* (7) subsequently used one of these, phenol, in a turbidimetric method for the estimation of serum lipids. Since the turbidity produced with γ-globulins is suppressed at elevated ionic strengths (7, 10, 151), the test was made more specific for serum lipids by the addition of 12% sodium chloride to the phenol reagent (7). This test was later modified by Polonovski *et al.* (165), who used 6% sodium chloride in their phenol reagent. These reagents have been used by a number of investigators and the turbidity produced appears to correlate to some degree with the total lipid and total cholesterol of serum (7, 165 to 181). Attempts have also been made, with some measure of success, to correlate the results of these methods with data obtained by paper electrophoretic procedures for the estimation of serum lipoproteins (167, 168, 177, 180, 182). It is possible that the phenol reagent which contains 6% sodium chloride is more sensitive to abnormal lipoproteins (165, 172); however, this observation should be investigated further with ultracentrifugal flotation procedures. Turbidity in a modified phenol test has been reported to be enhanced by the addition of heparin, and suppressed by the presence of free fatty acids (183). On the other hand, thymol turbidity appears to be suppressed by heparin (184).

The principal advantages of the thymol and phenol turbidity methods are simplicity, the small volume of serum required, and the availability of reagents. While temperature does affect the turbidity, the data can be readily corrected for this variable (185, 186). The major disadvantage of both phenol and thymol turbidity procedures is their incomplete specificity. They yield little information on the composition and concentration of the specific lipoprotein fractions altered in different disease states. Although they have been reported to yield results comparable to those obtained by methods employing sulfated polysaccharides (170, 178, 187), this correlation has been questioned (188). Furthermore, the turbidity obtained may be affected by the concentration of albumin (6, 154, 182), and fibrinogen and the products of fibrinolysis present in serum (189). Nevertheless, these procedures may have some application, within the limitations described, for following serum lipid levels in individuals during therapy.

PLASMA LIPOPROTEIN-SULFATED POLYSACCHARIDE COMPLEXES

The interaction of lipoproteins with sulfated polysaccharides may result in the formation of either soluble or insoluble complexes. Insoluble complexes have been studied by observing the development of turbidity or by the actual isolation and chemical analysis of the precipitate. Soluble complexes have been studied by moving boundary electrophoresis since the electrophoretic mobility of the lipoprotein-sulfated polysaccharide complex is greater than that of the free lipoprotein (190 to 193). Bernfeld *et al.* (190) found that the ascending and descending boundaries were both altered by the formation of a β-lipoprotein-sulfated pectic acid complex, whereas only the ascending boundary was altered with the formation of a β-lipoprotein-heparin complex (Table 2). These differences in electrophoretic behavior characterize complexes with little tendency to dissociate (irreversible complexes) and complexes with considerable tendency to dissociate (reversible complexes), respectively. Sugano (193) investigated soluble β-lipovitellin-heparin complexes and also found a tendency for complex dissociation in the descending limb during electrophoresis (Table 2). This tendency for dissociation may be expressed as the

TABLE 2. ELECTROPHORETIC MOBILITIES OBSERVED AS RESULT OF INTERACTION OF LIPOPROTEINS WITH SULFATED POLYSACCHARIDE

Buffer		Composition (mg/100 ml)		Mobilities × 10^5 (cm^2 sec^{-1} $volt^{-1}$)				Reference
pH	N			Ascending		Descending		
		β-Lipoprotein	Heparin					
8.6	0.1	980		3.0		3.0		(190)
8.6	0.1	980	80	4.5		3.1		
		β-Lipoprotein	Sulfated Pectic Acid					
8.6	0.1	980		3.0		3.0		(190)
8.6	0.1	980	80	4.9		4.2		
		β-Lipovitellin	Heparin	Fast	Slow	Fast	Slow	
9.8	0.1	800			5.7		5.3	
9.8	0.1		330	18.8		17.4		
9.8	0.1	800	330	18.3	7.8	16.2	5.5	
9.8	0.3	800			3.5		3.5	(193)
9.8	0.3	800	330	13.9	4.5	13.2	3.9	
7.8	0.25	800			1.25		1.2	
7.8	0.3*	800	330	13.6	3.35	13.0	2.05	

* Some precipitate formed. Electrophoretic analysis of the supernatant fraction.

reversible formation of a complex between the protein (P) and polyanion (A):

$$P + iA \underset{k_r}{\overset{k_f}{\rightleftharpoons}} PA_i$$

When the velocity constants for the forward (k_f) and reverse (k_r) reactions are both large, and the electrophoretic mobility of the polyanion is much greater than that of the protein, electrophoresis causes a depletion in the concentration of the former and dissociation of the complex (193, 194, 195). It is thus possible to distinguish between three types of molecular complex resulting from the interaction of lipoproteins and sulfated polysaccharides: (*a*) insoluble complexes; (*b*) reversible soluble complexes; (*c*) irreversible soluble complexes. The factors which affect the interaction of lipoproteins with sulfated polysaccharides include pH, ionic strength, and the presence of certain metal ions, as well as the nature of the polysaccharide and lipoprotein involved.

pH and Ionic Strength. Sulfated polysaccharides interact with many proteins on the acid side of their isoelectric points. Fibrinogen, albumin, and some lipoproteins, on the other hand, also form molecular complexes on the alkaline side of their isoelectric points. Serum, rather than plasma, is generally used in the investigation of lipoproteins in order to eliminate interference by fibrinogen-sulfated polysaccharide complexes. However, fibrinogen complexes are more soluble and more readily dissociated than lipoprotein complexes. Furthermore, certain sulfated polysaccharides selectively precipitate β-lipoproteins from plasma (191). Albumin forms soluble complexes with sulfated polysaccharides which do not interfere in precipitation methods for the isolation of lipoproteins (114, 115, 123 to 126).

Precipitation is a function of pH and the isoelectric point of the protein. The S_f 0–10 or β-lipoprotein fraction, isoelectric at pH 5.7 (196), forms a soluble complex with heparin in alkaline solutions and an insoluble complex at or below its isoelectric point (Table 3). The β-lipovitellin fraction of egg yolk, isoelectric at pH 5.9 (199), reacts in the same manner (Table 3). Since many other proteins precipitate in this pH range, reaction conditions must be adjusted for the selective precipitation of lipoproteins in the neutral to alkaline pH range. This is accomplished by the addition of specific metal ions to the reaction mixture, or by the utilization of sulfated polysaccharides which precipitate lipoproteins at an alkaline pH.

The specificity of some lipoprotein-sulfated polysaccharide interactions is a function of ionic strength. Thus heparin and calcium chloride flocculate the chylomicrons in lipemic serum but do not precipitate the β-lipoproteins unless the ionic strength is decreased (200, 201). Control of ionic strength is important in the selective precipitation of lipoproteins since other serum proteins may precipitate together with lipoprotein-sulfated polysaccharide-metal ion complexes at low ionic strengths (202). Insoluble lipoprotein-sulfated polysaccharide complexes dissolve in solutions of high ionic strength (37, 192, 201). This property

TABLE 3. EFFECT OF pH ON THE FORMATION OF LIPOPROTEIN-SULFATED POLYSACCHARIDE MOLECULAR COMPLEXES

Polysaccharide	Lipoprotein	pH	Molecular Complex	Reference
Heparin	β-Lipoprotein	8.6	Soluble-reversible	(190)
Heparin	β-Lipoprotein	5.0–5.7	Insoluble	(197)
Dextran sulfate*	β-Lipoprotein	8.6	Soluble-irreversible	(192)
Dextran sulfate*	β-Lipoprotein	5.0–5.7	Insoluble	(197)
Carrageenin-k	β-Lipoprotein	8.6	Soluble-irreversible	(192)
Carrageenin-k	β-Lipoprotein	6.8	Partially soluble	(198)
Carrageenin-k	β-Lipoprotein	4.6	Insoluble	(198)
Heparin	β-Lipovitellin	9.8	Soluble-reversible	(193)
Heparin	β-Lipovitellin	7.8	Partially soluble	(193)

* Low molecular dextran sulfate used as a synthetic anticoagulant.

of the molecular complex is important in many isolation procedures since the precipitate is isolated, dissolved in 5% to 12% sodium chloride, and reprecipitated by dilution with water or buffer (192, 201). The lipoprotein fraction may be purified by several reprecipitations.

Metal Ions. The formation of insoluble lipoprotein-sulfated polysaccharide complexes often depends upon the addition of metal ions to the reaction mixture. This effect has been investigated in detail by Burstein and his associates and the results of these studies are summarized in Table 4. Heparin and low molecular weight dextran sulfates both form soluble complexes with lipoproteins above pH 5.7 (192). Insoluble complexes are formed in the range from pH 6.5 to 8.5 by the addition of specific metal ions (206). The lipoprotein-sulfated polysaccharide-metal ion complexes are soluble above pH 9 (201, 206). Metal ions, pH, and ionic strength all contribute to the specificity of complex formation. For example, heparin forms a soluble complex with β-lipoproteins in the presence of Ca(II) or Mg(II), whereas chylomicrons in lipemic serum are flocculated under the same conditions (Table 4). An insoluble β-lipoprotein-heparin complex is formed with Ca(II) or Mg(II) if the ionic strength is lowered, but low molecular weight dextran sulfate precipitates β-lipoproteins in the presence of Ca(II) or Mg(II) without altering the ionic strength (Table 4). The γ-globulins are precipitated by heparin in the presence of Co(II), and Burstein and Prawerman (202) recently proposed a method for the turbidimetric estimation of γ-globulins in which β-lipoproteins are first precipitated in the presence of Ca(II), and then the γ-globulins are precipitated with Co(II).

The metal ions considered in Table 4 fall into two groups: (*a*) the alkali earths, Mg(II), Ca(II), Sr(II), and Ba(II), which interact primarily with carboxyl and sulfate groups, and (*b*) the transition elements, Mn(II), Co(II), and Ni(II), which are characterized by their strong tendency to form complexes with nitrogen containing ligands such as the amino and imidazole groups of proteins (214). The latter group of metal ions would be expected to form complexes with proteins and thereby contribute to the positive charge of the molecule. Thus the effect of these ions in promoting the formation of insoluble complexes with sulfated polysaccharides may be quite analogous to the effect of hydrogen ions. In this connection it

TABLE 4. EFFECT OF METAL IONS ON THE FORMATION OF INSOLUBLE SULFATED POLYSACCHARIDE MOLECULAR COMPLEXES IN SERUM AT pH 7.4*

Polysaccharide	Metal Ion	Composition of Insoluble Complex	Reference
Heparin	Ca(II), Mg(II)	β-Lipoprotein complex†	200, 201, 203, 204
Heparin	Ca(II), Mg(II)	Chylomicron complex	200, 201, 203, 204, 205
Heparin	Ni(II), Co(II), Mn(II)	β-Lipoprotein complex‡	200, 201, 203, 204, 206, 207, 208
Dextran sulfate	Ca(II), Mg(II)	β-Lipoprotein complex	200, 201, 203, 204, 207 to 212
Dextran sulfate	Ba(II), Sr(II)	Dextran sulfate-metal ion complex	204
Mepesulfate®,§	Ca(II)	S_f 0–10 and S_f 10–400 lipoprotein complex	213

* The reaction mixtures were buffered by serum; however, the exact pH was not measured in every experiment.

† An insoluble complex formed only at low ionic strength.

‡ Other serum proteins also precipitate at low ionic strength (200, 203).

§ Mepesulfate® is the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside obtained from Hoffmann-La Roche.

should be noted (Table 4) that heparin in the presence of these ions precipitates other serum proteins as well as β-lipoproteins at low ionic strength.

Although the addition of metal ions may be necessary for the precipitation of β-lipoproteins by some sulfated polysaccharides, their presence does not appear necessary for precipitation by others. Bernfeld *et al.* (190, 191, 192) precipitated lipoproteins from serum with several sulfated polysaccharides without adding metal ions; however the Ca(II) already present in serum might have been sufficient. Florsheim and Gonzales (213) found that whereas it was necessary to add calcium ions to effect the precipitation of β-lipoproteins with Mepesulfate®, no addition of calcium ions was necessary with rice starch sulfate. Oncley *et al.* (37) precipitated low density lipoproteins from resin collected plasma with a high molecular weight dextran sulfate. Although the calcium ion concentration was probably very low in this case, analytical data on this point are not available. Insoluble lipoprotein-sulfated polysaccharide-metal ion complexes are readily dissociated by the addition of chelating agents such as oxalate or citrate (201, 206), by increasing the ionic strength (37, 192, 202, 204, 206), and by selectively precipitating the sulfated polysaccharide as a Ba(II), Sr(II), or protamine salt (192, 202, 204, 206). It is of interest to note here that Hoch and Chanutin (126) found that chelating agents did not affect the formation of soluble protein-heparin complexes as studied electrophoretically.

Structure of the Sulfated Polysaccharide. Bernfeld *et al.* (192) found that sulfate content and degree of polymerization are two major factors influencing complex formation. β-Lipoprotein is precipitated completely by sulfated corn amylopectin, which contains from 0.85 to 2.18 sulfate groups per hexose unit, while only partial precipitation is obtained with 0.22 to 0.57 sulfate groups per hexose unit (Table 5). Soluble complexes are not formed with corn amylopectins of low sulfate content. It thus appears that complexes, if formed at all with sulfated corn amylopectins, are of the insoluble variety. The effect of sulfate content was confirmed with sulfated hyaluronic acid and sulfated chondroitin sulfate, both of which form stronger complexes than the native polysaccharides (192). Precipitation is enhanced by increasing the concentration of polysaccharides with low sulfate content.

Insoluble complexes are formed with high molecular weight dextran sulfates, whereas soluble-irreversible complexes are formed with low molecular weight dextran sulfates with the same sulfate content per repeating unit (Table 5). Corn amylose sulfates varying in degree of polymerization from 80 to 800 demonstrated a moderate transition from soluble-irreversible to insoluble types. It is of interest to note that precipitation is not increased by increasing the concentration of low molecular weight polymer.

Bernfeld *et al.* (192) also found that the chemical composition of the polysaccharide influenced the nature of the molecular complex. Sulfated polysaccharides which contain carboxyl groups (sulfated pectic or polymannuronic acids, Table 5) form only soluble-irreversible complexes. The molecular complex is of the soluble-reversible type in the case of sulfated poly-

TABLE 5. EFFECT OF POLYSACCHARIDE STRUCTURE ON THE FORMATION OF MOLECULAR COMPLEXES WITH β-LIPOPROTEINS AT pH 8.6*

Polysaccharide	Sulfate Groups per Repeating Unit	Degree of Polymerization	Molecular Complex
Corn amylopectin sulfate	2.18	>2,000	Insoluble
Corn amylopectin sulfate	0.85	>2,000	Insoluble
Corn amylopectin sulfate	0.57	>2,000	Insoluble†
Corn amylopectin sulfate	0.22	>2,000	Insoluble†
Corn amylopectin	0	>2,000	None
Dextran Sulfate, from Strain			
NRRL-B512	1.44	240,000	Insoluble
NRRL-B512	1.58	440	Soluble-irreversible‡
NRRL-B1254	1.59	>6,000	Insoluble
NRRL-B1254	1.09	540	Soluble-Irreversible‡
Pectic acid sulfate	1.4		Soluble-Irreversible
Polymannuronic acid sulfate	1.15		Soluble-Irreversible
Hyaluronic acid sulfate	0.58–1.33		Soluble-Reversible
Chitin sulfate	0.68		Soluble-Reversible
Heparin	1.45–2.68		Soluble-Reversible

* Compiled from the investigation of Bernfeld *et al.* (192).

† Incomplete precipitation was obtained; however, no soluble complex was formed.

‡ A slight turbidity developed.

saccharides containing N-acetyl and N-sulfatyl hexosamine residues. This is illustrated by sulfated hyaluronic acid, sulfated chitin, and heparin in Table 5.

The polysaccharides suitable for complex formation have no apparent specific requirement for degree of branching, branching points, configuration of glycosidic linkages, or hexose composition (192). However, this investigation was limited to a single pH, and metal ion interactions were not considered. Other relationships may become apparent when pH, ionic strength, and metal ion effects are considered along with polysaccharide structure.

Lipoprotein Specificity. Many of the experimental results obtained in the study of plasma lipoprotein-sulfated polysaccharide interactions are difficult to interpret because the criteria used to establish specificity were inadequate. In precipitation experiments specificity was usually demonstrated by paper electrophoresis (200 to 212). Paper electrophoresis may show the absence of β-lipoproteins from the supernatant solution. However, chylomicrons and S_f 10–400 lipoproteins may not be differentiated from one another by this technique. Groulade *et al.* (215) were able to correlate turbidity or precipitation with paper electrophoretic data by combining the β-lipoproteins and the less mobile lipoproteins as one fraction. This indicates that precipitation involves both S_f 0–10 and S_f 10–400 lipoproteins, though the correlation was less apparent with lipemic sera (215). Moving boundary electrophoresis indicates that serum lipoprotein-sulfated polysaccharide interactions involve the β-lipoprotein or S_f 0–10 lipoprotein fraction (190, 191, 192, 216). Starch gel and agar electrophoretic data are difficult to interpret since molecular size affects protein mobility in a starch gel (217), and agar, a sulfated polysaccharide, interacts with the low density lipoproteins (218).

Analytical and preparative ultracentrifugation have been used by several investigators to determine lipoprotein specificity in sulfated polysaccharide interactions. Oncley *et al.* (37) fractionated the lipoproteins from a high molecular weight dextran sulfate complex by ultracentrifugation in a density gradient. Analytical ultracentrifugation was then used to measure the S_f distribution in the lipoprotein fractions. These fractions contained S_f 3–9, S_f 5–15, and S_f 10–100 lipoproteins; however, the quantitative recovery of the different low density lipoproteins from serum was not measured. Florsheim and Gonzales (213) compared serum and the lipoproteins obtained as an insoluble rice starch sulfate complex by analytical ultracentrifugation. The precipitate contained the S_f 0–12 lipoproteins and most of the S_f 12–400 lipoproteins. Boyle and Moore (198) fractionated serum by preparative ultracentrifugation into the lipoprotein-free infranate ($d > 1.21$), high density lipoproteins (d 1.063–1.21), S_f 0–16 lipoproteins (d 1.006–1.063), and $S_f > 17$ lipoproteins ($d < 1.006$). Only the S_f 0–16 lipoprotein fraction formed an insoluble complex with the carrageenin used in their study. Sulfated corn amylopectin precipitated S_f 2–12 lipoproteins from serum, according to a study by Bernfeld *et al.* (192). In an earlier study Bernfeld *et al.* (216) found the turbidity with sulfated corn amylopectin to be specific for the β-lipoprotein fraction as isolated by zone electrophoresis on a starch block.

An insoluble complex has been obtained by the flocculation of lipemic serum with heparin and Ca(II) ions, and attributed to the chylomicron fraction (201, 203, 204, 207, 208). Since the chylomicron fraction is often poorly defined, and since lactescent serum also contains increased amounts of S_f 10–400 lipoproteins, ultracentrifugation studies are necessary to confirm this important observation. Burstein and Samaille (205) prepared antibodies against this chylomicron fraction and found them to flocculate chylomicrons, whereas they only reacted weakly with β-lipoproteins. However, the chylomicrons employed in the production of antibodies were subject to centrifugation during the isolation procedure from the heparin complex and may therefore have been separated from any S_f 10–400 lipoproteins coprecipitated with them. Burstein and Samaille (201) also found that the protein content of the lipoprotein-heparin-Ca(II) ion precipitates was altered by varying the ionic strength of the reaction mixture. The fraction isolated at low ionic strength contained more protein than the fraction isolated directly from serum. To what extent this difference indicates contamination by nonlipoprotein protein or selectivity between precipitation conditions for the chylomicrons, S_f 10–400 lipoproteins, and S_f 3–9 lipoproteins remains to be investigated.

Electrophoretic and ultracentrifugal techniques indicate that the sulfated polysaccharide interactions with respect to human serum are specific for chylomicrons and low density lipoproteins. This is further substantiated by the more sensitive immunochemical techniques. Burstein and Oudin (219) prepared antibodies against the lipoproteins precipitated from serum as a dextran-Ca(II) complex, and demonstrated the immunochemical homogeneity of the lipoprotein fraction. Antibodies to this fraction have been used to estimate the β-lipoprotein content of serum (220, 221). The antibodies are probably not strictly specific for β-lipoproteins alone, since S_f 0–10 lipoproteins and S_f 10–400 lipoproteins do not differ immunochemically (57). As discussed earlier, there is some question concerning the electrophoretic behavior of S_f 10–400 lipo-

proteins; at least some portions of this fraction do not migrate as β-lipoproteins. Briner *et al.* (57) isolated low density lipoproteins by precipitation with high molecular weight dextran sulfate, fractionated the precipitated lipoproteins in a saline density gradient into S_f 0–10 and S_f 10–400 lipoproteins, and prepared antibodies against the two fractions. These two fractions cross-reacted completely, and therefore cannot be distinguished by immunochemical means.

Although the composition of different lipoprotein fractions isolated from normal sera by other procedures has been studied in detail, chemical analysis has not been used extensively in the characterization of lipoprotein-sulfated polysaccharide precipitates. This is due in part to the fact that the precipitates often contain mixtures of S_f 0–10 and S_f 10–400 lipoproteins, and the composition of the precipitate may vary, depending on the relative concentration of these two lipoproteins in the serum. The total lipid content of sulfated amylopectin precipitates (190) and the range in protein, sterol, phosphatide, and glycerol content for a low molecular weight dextran sulfate precipitate (206) have been reported. Polonovski *et al.* (211) studied the total lipid, cholesterol, and phospholipid composition of a dextran sulfate precipitate and the corresponding supernatant solution. Cholesterol to phospholipid ratios calculated from this study indicate that the precipitate contained low density lipoproteins and the supernatant contained the high density lipoprotein fraction. Castaigne and Amselem (212) measured the total cholesterol, esterified cholesterol, and free cholesterol in similar fractions and found the appropriate esterified to total cholesterol ratios for low and high density lipoproteins in the precipitate and supernatant, respectively. Oncley *et al.* (37) isolated S_f 10–100 and S_f 3–9 lipoprotein fractions from a high molecular weight dextran sulfate precipitate by ultracentrifugal flotation and showed that the composition of these fractions was identical with that of lipoprotein fractions isolated directly by ultracentrifugation. The cholesterol distribution between lipoprotein-sulfated polysaccharide precipitate and supernatant solution has been measured in several studies (178, 206, 208, 209, 222), and is similar to the cholesterol distribution between low and high density lipoproteins as determined by ultracentrifugal, electrophoretic, and cold-ethanol fractionation methods. However, Beaumont and Beaumont (223) found an appreciable quantity of vitamin A in lipoprotein-dextran sulfate-Ca(II) precipitates, whereas Krinsky *et al.* (224) found only traces of vitamin A in lipoprotein-dextran sulfate precipitates further purified by ultracentrifugal flotation.

Sulfated polysaccharide precipitation methods have been used to investigate the plasma lipoprotein distribution in disease states associated with hypercholesterolemia and hyperlipemia (170, 172, 173, 178, 180, 181, 198, 208, 209, 211, 212, 216, 222, 225, 226, 227). These studies are difficult to evaluate since the methods are usually standardized with sera from normal subjects, or lipoprotein fractions isolated from normal subjects. Since the S_f distribution and composition of lipoprotein fractions show wide variation in many diseases, direct extrapolation from normal to pathological sera may be misleading. The cholesterol distribution between precipitate and supernatant observed in hyperlipemic sera is generally similar to that obtained by other methods, although in some cases the cholesterol content of the supernatant (high density lipoprotein) is greater than would be expected from other studies (178, 209, 211, 212, 222). It is possible that all portions of the low density lipoprotein spectrum are not precipitated under the conditions employed. It is also important to be certain that the concentration of precipitating polyanion is adequate for quantitative precipitation.

The investigation of plasma lipoproteins in different animal species by sulfated polysaccharide precipitation methods presents special problems. Castaigne and Amselem (212) used dextran sulfate to investigate the cholesterol distribution in a number of animal species. Their results were similar to those obtained by other methods; however, the characterization of animal lipoproteins is as yet too incomplete for a significant correlation between methods. Burstein and Samaille (228) studied lipoprotein-dextran sulfate complexes in several animal species by paper electrophoresis and were able to demonstrate precipitation of β-lipoproteins. However, Florsheim and Gonzales (213) obtained variable precipitation of different animal β-lipoproteins with rice starch sulfate, a polyanion which precipitates human low density lipoproteins quantitatively. These workers found that Mepesulfate® precipitated many animal β-lipoproteins in a quantitative manner. The use of sulfated polysaccharides will undoubtedly contribute to the characterization of animal serum lipoproteins, but it is most important that they be initially employed in conjunction with electrophoretic and ultracentrifugal techniques.

Concentration. The solubility of lipoprotein-sulfated polysaccharide complexes is influenced by the relative concentration of lipoprotein and sulfated polysaccharide. Soluble complexes are formed if either lipoprotein or sulfated polysaccharide is present in large excess. Oncley *et al.* (37) suggested that a serum aliquot be titrated to maximum turbidity in order to establish the optimal sulfated polysaccharide concentration for

precipitation. Bernfeld *et al.* (216), Boyle and Moore (198), and Antoniades *et al.* (226) have published experimental results relating precipitation or turbidity to sulfated polysaccharide concentration. The region of maximum precipitation is broad, and a single polyanion concentration is adequate for most studies. Nevertheless, this region should be determined particularly in the evaluation of new sulfated polysaccharides or in the isolation of lipoproteins from markedly hypercholesterolemic or hyperlipemic sera.

Isolation of Plasma Low Density Lipoproteins. Several lipoprotein isolation procedures involving the use of sulfated polysaccharides have been reported and three of these are outlined in Charts I, II, and III. In two procedures (Charts I and II) the lipoproteins are purified by reprecipitation of the sulfated polysaccharide complex and the low density lipoproteins are isolated as a single fraction. In the third procedure (Chart III) density gradient ultracentrifugation is employed to further purify the lipoproteins and at the same time separate them into S_f 0–10 and S_f 10–400 fractions. The sulfated polysaccharide is removed either as an insoluble barium salt (Chart I), or an insoluble protamine complex (Chart II), or is concentrated in the infranatant solution by ultracentrifugation (Chart III). These methods are amenable to modification, and certain steps such as reprecipitation and ultracentrifugation may be employed in the same procedure. In studies where the rapid isolation of low density lipoproteins is required and purity is not critical, a

CHART I

ISOLATION OF β-LIPOPROTEINS WITH SULFATED AMYLOPECTIN (192)

Serum 60 ml

a. Add 9 ml 1% sulfated amylopectin
b. Centrifuge at 15,000 × g

Precipitate	Supernatant
β-Lipoprotein-sulfated amylopectin complex	Discard

a. Dissolve in 3 ml 12% NaCl
b. Reprecipitate by diluting with 57 ml 0.02 M Na_2HPO_4
c. Centrifuge at 15,000 × g

Precipitate	Supernatant
β-Lipoprotein-sulfated amylopectin complex	Discard

a. Dissolve in 3 ml of 12% NaCl
b. Add 0.75 ml 0.1 M barium acetate
c. Centrifuge

Precipitate	Supernatant
Amylopectin sulfate-Ba (II) complex	β-Lipoproteins

a. Pass through Amberlite® IR 120* column charged with NaCl to remove excess Ba (II)†

β-Lipoprotein solution‡

* Amberlite® IR 120 obtained from Rohm and Haas.

† Lipoprotein stability is improved if excess Ba (II) is precipitated by the addition of 1 ml 0.1 M sodium sulfate rather than passage through an ion-exchange column. (P. Bernfeld, private communication.)

‡ The β-lipoprotein solution is stabilized by the addition of ethylenediaminetetraacetic acid, 2 mg/10 ml. or pyrophosphate, 0.01 M final concentration.

CHART II

ISOLATION OF β-LIPOPROTEINS WITH DEXTRAN SULFATE (201)

Serum 100 ml

a. Add 2 ml 10% dextran sulfate* and 10 ml 1 M $CaCl_2$
b. Centrifuge at 5450 × g for 10 minutes†

Precipitate	Supernatant
β-Lipoprotein-dextran sulfate complex	Discard

a. Dissolve in 10 ml 5% NaCl
b. Reprecipitate by diluting with 90 ml distilled water and 10 ml 1 M $CaCl_2$
c. Centrifuge at 5450 × g for 10 minutes†

Precipitate	Supernatant
β-Lipoprotein-dextran sulfate complex	Discard

a. Dissolve in 5 ml of 0.1 M sodium oxalate
b. Add 2 g NaCl and 2.5 ml 2% protamine sulfate‡
c. Centrifuge

Precipitate	Supernatant
Dextran sulfate-protamine complex	β-Lipoprotein solution

a. Dialyze against 0.15 M NaCl

β-Lipoprotein solution

* Dextrarine® obtained from Equilibre Biologique.
† M. Burstein, private communication.
‡ High ionic strength prevents formation of a protamine-lipoprotein complex.

single precipitation step may be adequate for the isolation of a low density lipoprotein fraction (229, 230). The purified lipoprotein fractions should be analyzed for the presence of residual sulfated polysaccharides. This may be accomplished by metachromatic staining with toluidine blue (231) or the thiazine dye Azure A (232), or by measuring the antithromboplastic activity of the fraction (232). Ribonuclease inhibition has been used as a sensitive assay for heparin (233). Jaques and Bell (234) have recently discussed the methods used for the determination of heparin.

It should perhaps be re-emphasized that isolation procedures developed for use with normal human serum may require modification before they are applied to the sera of hyperlipemic individuals or of other animal species. For example, the insoluble complexes obtained from hyperlipemic sera may float rather than sediment upon centrifugation (201, 203, 205, 206, 208, 211). In adapting these methods to other than normal human sera, preliminary studies including electrophoretic, ultracentrifugal, and chemical analyses of both precipitate and supernatant fractions should be carried out. It may be necessary to alter certain of the parameters which affect specificity and quantitative precipitation such as pH, ionic strength, metal ions, and concentration of precipitant.

Quantitative Estimation of Plasma Lipoproteins. Several procedures involving the formation of insoluble sulfated polysaccharide complexes have been proposed for the estimation of plasma lipoproteins. In some of these the cholesterol content of precipitate and supernatant fractions is determined (178, 203, 207, 208, 209, 212). In others the turbidity produced is measured as an estimate of the amount of insoluble lipoprotein complex formed (170, 178, 188, 191, 203, 206, 210, 215, 216, 225, 226). Both methods have limitations. The cholesterol content of the precipitate and the turbidity give an estimate of the total low density lipo-

proteins. No information is obtained concerning the relative amounts of the major low density lipoprotein fractions. This defect could perhaps be partially remedied by determining the lipid phosphorus content of the precipitated complex in addition to the cholesterol. Since the cholesterol to phospholipid ratios of the S_f 0–10 and S_f 10–400 subfractions differ considerably (1.30–1.45 and 0.78–0.90, respectively), some idea of the relative contribution of each to the total might be obtained. It should be kept in mind, however, that the cholesterol to phospholipid ratios of the S_f 0–10 and S_f 10–400 lipoproteins are frequently altered in abnormal states. Turbidimetric methods are also subject to error dependent upon the relative contribution of S_f 0–10 and S_f 10–400 lipoproteins since the turbidity increment is probably different for the two fractions. Lactescent sera exhibit an inherent turbidity which interferes with evaluation of the turbidity due to complex formation. Furthermore, turbidity produced by abnormal lipoproteins may not be comparable to that

CHART III

ISOLATION OF LOW DENSITY LIPOPROTEIN FRACTIONS WITH DEXTRAN SULFATE (37, 57)

Serum

a. Add dextran sulfate*
(Titrate aliquot to maximum turbidity)
b. Stir 2 hours at 2°
c. Centrifuge 30 minutes 1000 × g at 2°

Supernatant		Precipitate
Discard		Low density lipoprotein-dextran sulfate complex
		a. Dissolve in 5 ml 2 M NaCl† per 200 ml original serum b. Prepare density gradient tube
Gradient Tube A 13.5 ml lusteroid tube a. Place 6 ml lipoprotein solution in tube b. Fill tube by layering 0.15 M NaCl† above lipoprotein	Centrifuge§ 20 hours 100,000 × g, 2° to 5°	Gradient Tube A‡ a. Top layer: turbid S_f 10–400 lipoproteins b. Intermediate layer: S_f 5–15 lipoproteins c. Middle layer: orange S_f 0–10 lipoproteins d. Infranate: protein contaminants and dextran sulfate
Gradient Tube B 13.5 ml lusteroid tube a. Dialyze S_f 0–10 lipoproteins against 20 volumes 2 M NaCl† for 18 hours b. Place 6 ml lipoprotein solution in tube c. Fill tube by layering 0.15 M NaCl† above lipoproteins	Centrifuge§ 20 hours 100,000 × g, 2° to 5°	Gradient Tube B‡ a. Top layer: discard b. Middle layer: orange S_f 0–10 lipoproteins c. Bottom layer: discard Dialyze S_f 0–10 lipoproteins against 0.15 M NaCl†
Centrifugation C 13.5 ml lusteroid tube a. Dialyze S_f 10–400 lipoproteins against 20 volumes 0.15 M NaCl† for 18 hours b. Fill tube	Centrifuge§ 20 hours 100,000 × g, 2° to 5°	Centrifugation C‡ a. Top layer: turbid S_f 10–400 lipoproteins# b. Infranate: small amount S_f 10–20 lipoproteins

* High molecular weight dextran sulfate (37).

† All salt solutions contain 0.1 g per liter of the disodium salt of ethylenediaminetetraacetic acid adjusted to pH 7.0 ± 0.2 with 1 N NaOH.

‡ Separate layers with tube cutter.

§ Spinco Model L ultracentrifuge.

Chylomicrons and lipoproteins with a high S_f may be separated by centrifugation for a shorter time in a lower centrifugal field.

produced by a like amount of normal lipoprotein. Finally, it should be pointed out that purified lipoproteins have not proved satisfactory as standards for the turbidimetric analysis of serum lipoproteins, largely because a number of substances present in serum enhance turbidity (192).

Molecular Complexes in Plaque Formation. The demonstration of synthetic lipoprotein-sulfated polysaccharide complexes led Gerö *et al.* (235) to suggest that lipid deposition in the aorta resulted from the formation of a plasma lipoprotein-mucopolysaccharide complex at the site of plaque formation. Atherosclerosis is accompanied by an elevation in aortic mucopolysaccharides (235, 236, 237), and there may be a focal increase in metachromatic material in the area of plaque formation (238). Farquhar *et al.* (239) have suggested that atheroma and plasma fatty acids are in a dynamic equilibrium; however, the net deposition of lipid or preformed plasma lipoprotein in the aorta has not been demonstrated. Also, lipid synthesis has been shown to occur in arterial tissue (240). The relative contribution of plasma lipid and synthesis of lipid, *in situ*, to the lipid in the atherosclerotic lesion, as well as the nature of the binding of the lipid therein, remains obscure (241).

THE INTERACTION OF LIPOPROTEINS WITH NEUTRAL POLYMERS

Polyvinylpyrrolidone (PVP) is a neutral polymer which has been used as a plasma extender.[5] Although PVP preparations are available in several molecular weight ranges, the one studied most contains between 150 and 170 monomer units and has a mean molecular weight of about 40,000 (242). PVP as obtained from the manufacturer migrates in an electric field and exhibits two types of ionizable groups (pK 4.3 and 10) on titration with alkali (243). However, it has been shown that these groups are associated with a low molecular weight inpurity and PVP which has been subjected to exhaustive dialysis is essentially nonionogenic throughout the pH range 2.5 to 10 (244). Also, ultraviolet absorption indicates that the structure of the polymer remains essentially unaltered over a wide pH range (245, 246). PVP forms molecular complexes with a number of compounds (242, 244, 245, 247, 248). Higuchi and Kuramoto (247) have suggested that complex formation is effected by hydrogen bonding. Another neutral polymer, poly-N-vinyl-5-methyl-2-oxazolidinone (Devlex®), has binding properties similar to PVP, and this has been attributed to the formation of resonance complexes.[6]

The literature on the interactions between PVP and serum proteins appears to be contradictory. May *et al.* (242), Ardry (249), and Hirsch and Cattaneo (250) found no evidence of interaction by electrophoretic techniques. However, Grabar (251) concluded from the results of hemagglutination studies that PVP did interact with serum proteins. Furthermore, Ramos (252) reported that PVP altered the electrophoretic mobilities of albumin and γ-globulins. Banerjee (253) studied the interaction between PVP and fibrinogen by measuring viscosity effects and found that high molecular weight PVP formed complexes with fibrinogen, whereas low molecular weight PVP did not appear to do so. It is therefore possible that some of the apparent discrepancies are due to differences in the PVP preparations used by the several investigators.

In 1957 Burstein (5) reported that PVP formed an insoluble complex with the β-lipoproteins. Burstein and co-workers (5, 254, 255, 256) have studied this interaction in detail. Precipitation with PVP is a function of pH, ionic strength, and PVP concentration. Only chylomicrons complex above pH 9.5, whereas β-lipoproteins also form precipitates in the intermediate pH range, and both lipoproteins and γ-globulins precipitate when the pH is lowered to 4 (5, 254, 255). The formation of insoluble complexes with β-lipoproteins and γ-globulins is inhibited in hypertonic sodium chloride solutions (5, 254, 255). Hypertonic salt solutions do not inhibit PVP interaction with chylomicrons or the flocculation of chylomicron-PVP complexes (254, 255). Only chylomicrons are flocculated when the final PVP concentration is 5%, both chylomicrons and β-lipoproteins are precipitated when the concentration is raised to 8%, and other globulins are also precipitated in 12% PVP (5, 254, 256). The lipovitellenin or rising lipoprotein fraction of egg yolk may be flocculated as a Devlex-130® complex.[7]

PVP has been employed in a turbidimetric method for the estimation of chylomicrons and lipoproteins in serum (256). Burstein and Prawerman (255) recently developed a method for the isolation of low density lipoproteins from hyperlipemic sera which involves varying ionic strength and PVP concentration for the selective formation of insoluble lipoprotein-PVP complexes (Chart IV). The lipoprotein fractions are further purified as heparin complexes, and the chylomicrons isolated by a final ultracentrifugal flota-

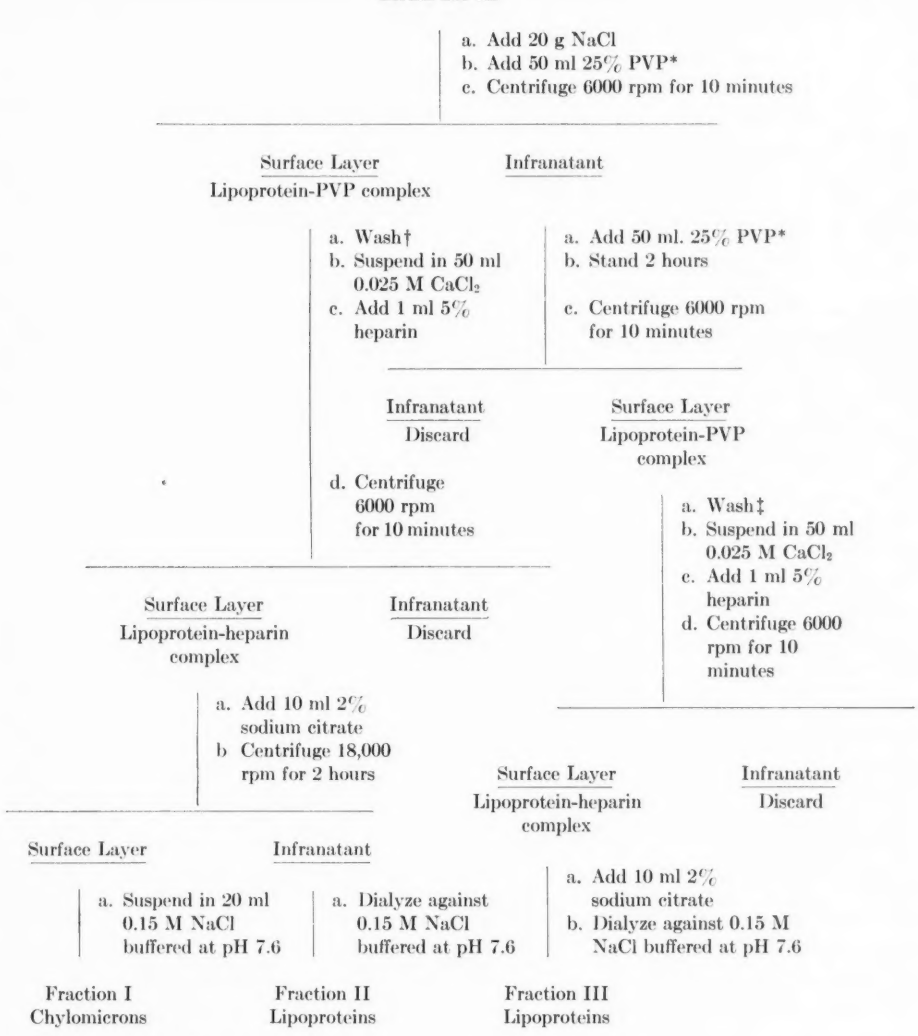
[5] See footnote 3.

[6] See footnote 4.

[7] D. A. Wolfe and D. G. Cornwell, unpublished observations.

CHART IV

ISOLATION OF LOW DENSITY LIPOPROTEINS FROM HYPERLIPEMIC SERUM WITH PVP (255)



* Subtosan retard® obtained from Specia.
† Wash 3 times by suspending in 50 ml of a solution containing 10% NaCl and 5% PVP.
‡ Wash 3 times by suspending in 50 ml of a solution containing 10% NaCl and 8% PVP.

tion step. The composition of the three fractions obtained is summarized in Table 6. The lactescence of the three fractions (comparing aliquots with equal lipid content) decreases from fraction I to fraction III. Fraction III is more opalescent than the β-lipoprotein fraction obtained from normal serum. It would appear from this study that considerable specificity can be attained within the low density lipoprotein spectrum with precipitation techniques employing neutral polymers. However, much work remains to be done in defining conditions and characterizing the fractions obtained.

In summary, the fractionation, estimation, and characterization of lipoproteins by complex formation with a variety of high molecular weight substances is an active area of research. However, although the formation of insoluble molecular complexes has proved valuable in the initial concentration and purification of certain

TABLE 6. COMPOSITION OF LIPOPROTEIN FRACTIONS ISOLATED FROM LIPEMIC SERUM BY PRECIPITATION WITH PVP

Lipoprotein Fraction	Percentage Composition (Range)*		
	Protein	Cholesterol	Phospholipid
I	1.3–2.0	4.4–6.4	7.4–8.8
II	3.8–5.2	7.2–10.1	9.5–11.0
III	6.3–7.0	11.0–12.6	14.0–15.4
β-Lipoproteins†	16–20	26–33	14–20.5

* Calculated from the data of Burstein and Prawerman (255).
† Lipoproteins isolated from the serum of a normal subject.

lipoprotein classes, our knowledge and methodology have not yet progressed sufficiently to employ these methods to the exclusion of others for the preparation of homogeneous lipoprotein fractions. Ultracentrifugal procedures remain the method of choice for the isolation and estimation of specific lipoproteins.

ADDENDUM

Several important studies have appeared since the completion of this review. Bernfeld *et al.* (257) have employed sulfated amylopectin in a nephelometric procedure for the estimation of relative serum lipoprotein concentrations in normal as well as disease states. They describe a method for the standardization of sulfated amylopectin preparations based on the turbidity produced in the formation of sulfated amylopectin-Ba(II) complexes. Ledvina *et al.* (258), using heparin in a turbidimetric procedure, have published a study on lipoprotein concentrations in thyroid disorders. Amenta and Waters (259) have isolated a mucopolysaccharide from human aortas and used this sulfated polysaccharide to precipitate lipoprotein fractions obtained from the serum of hypercholesterolemic rabbits. Amenta and Waters (260) have also investigated the formation of insoluble molecular complexes between rabbit lipoprotein fractions and gelatin.

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Separation of lipid classes by chromatography on florisil*

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SUMMARY

Chromatography on Florisil® (activated magnesium silicate) was used to separate model compounds representative of hydrocarbons, cholesterol esters, triglycerides, free sterols, diglycerides, monoglycerides, and free fatty acids. The order of elution was the same as that observed in silicic-acid chromatography except that free fatty acids were eluted after monoglycerides. Recoveries were nearly quantitative and the positions of individual compounds on the chromatograms were highly reproducible. Phospholipids were not eluted under the conditions used for separating the above compounds, and were eluted less readily from Florisil than from silicic acid with methanol. Florisil had definite advantages over silicic acid for the separation of lipid classes by column chromatography. It required no prewashing or other pretreatment except deactivation with water. Columns were quickly and easily packed, and the relatively coarse mesh of the Florisil permitted rapid flow rates. Separations could be achieved in much shorter times with smaller volumes of eluting solvents. Preliminary experiments indicated that chromatography on Florisil gave good separations of lipid classes of naturally occurring lipids extracted from liver and blood.

Current interest in the metabolism of lipids has indicated a need for chromatographic methods of separating the various classes of lipids which occur in nature. Chromatography on silicic acid has been widely used for this purpose since its introduction by Trappe (1, 2) and Borgström (3, 4) but, although it is capable of separating many classes of lipids (5, 6, 7), it has certain disadvantages, and the separations are not always reliable unless particular care is taken to ensure that conditions are duplicated exactly (7).

Chromatography on Florisil® was used in earlier experiments in this laboratory for the separation of fecal sterols from other fecal lipids (8). It had also been previously employed by other workers for the separation and purification of a number of different types of lipids such as tocopherols (9), steroid hormones (10), cerebrosides (11), and fatty acid esters (12). In the course of our experiments it became apparent that the adsorption characteristics of Florisil for classes of neutral lipids were somewhat similar to those of silicic acid. Accordingly, a series of chromatograms was run on Florisil columns with pure reference compounds in order to compare the results with those obtained by other workers using silicic acid. The successful separation of reference compounds led to further experiments on the separation of classes of naturally occurring lipids from rat liver and blood.

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METHODS

Florisil consists of hard, porous, white granules whose composition is reported to be as follows: magnesium oxide 15.5 ± 0.5%, silicon dioxide 84.0 ± 0.5%, and sodium sulfate 0.5% average (1.0% maximum).[1] It was obtained from the Floridin Co. in the form of 60- to 100-mesh material activated at 650°.[2] Material of 100 to 200 mesh was tested in a few experiments, but the coarser grade seemed preferable. Silicic acid (suitable for chromatographic analysis) was obtained from the Mallinckrodt Chemical Works, and Hyflo Super-Cel from the Johns-Manville Co., Ltd.

[1] This information is contained in a bulletin obtained from the Floridin Co., Tallahassee, Fla., on the properties and uses of Florisil as a chromatographic adsorbent.

[2] Florisil activated at 260° or at 110° may also be obtained from the Floridin Co. Chromatographic separations very similar to those described here were obtained by using Florisil activated at 260° without subsequent addition of water. Lipids such as cholesterol palmitate, tripalmitin, and cholesterol were also completely separated on Florisil activated at 110°, but the compounds were eluted somewhat earlier than in the experiments described.

Skellysolve B (a mixture of hydrocarbons, mainly *n*-hexane, boiling at 60°–71°) was obtained from Merck and Co. It was dried over sodium and distilled, and the portion boiling at 68°–70° was used. The purified Skellysolve B will hereafter be referred to as hexane. Merck reagent methanol and chloroform and Mallinckrodt anhydrous ether were used without further purification.

Standard reference compounds were obtained as follows: Paraffin (refined, m.p. 53°–55°)—A. H. Thomas Co.; squalene—Matheson, Coleman and Bell; cholesteryl palmitate—a gift of Dr. Leon Swell, Veterans Administrative Center, Martinsburg, West Virginia; tripalmitin—Hormel Institute, Austin, Minnesota; cholesterol—a commercial preparation purified by two recrystallizations from ethanol; 1,2-dipalmitin and 1-monopalmitin—gifts of Dr. F. H. Mattson, Procter and Gamble Co., Cincinnati, Ohio; palmitic acid—Nutritional Biochemicals. Rat liver and blood lipids were obtained by repeated extraction with ethanol-ether 3/1 (v/v) at room temperature. The extracts were concentrated *in vacuo* and the crude lipids were re-extracted from the concentrate with petroleum ether and dried over sodium sulfate.

Column Preparation. The initial experiments were carried out with a column containing 30 g of Florisil and measuring 2.0 cm in diameter × 17 cm in length. It was prepared by filling the chromatographic tube with hexane and adding the dry Florisil in portions, tapping the tube between additions to ensure even packing. Later experiments were carried out with a 12 g column, measuring 1.2 cm × 15 cm, prepared in the same way.

The early experiments were performed with a sample of Florisil which had been in the laboratory for several years. This gave satisfactory separations when used without preliminary treatment, but when similar separations were attempted with a fresh supply of Florisil, it was found that the different lipid classes were eluted much later in the chromatogram. This indicated that the new Florisil had a greater adsorption strength. It was therefore deactivated by mixing with a measured volume of water in a glass-stoppered round-bottom flask, shaking until the mixture was free-flowing, and allowing to stand overnight to equilibrate. It was then packed in a column as described above. The addition of 7 ml of water per 100 g of Florisil gave material which had chromatographic properties similar to the Florisil used in the initial experiments.

Chromatographic Procedure. When all the Florisil had been added to the column, the excess hexane was allowed to flow out until the surface of the liquid reached the top of the packed column, and the lipids to be separated were added to the top of the column in a small volume of hexane. The head of liquid was again allowed to drop to the top of the column, and elution was carried out as described for individual experiments. Reference compounds such as mono- and diglycerides, which were relatively insoluble in hexane, were added as dry powders to the top of the column.

During elution of the columns bubbles of solvent vapor occasionally formed, and in some cases caused actual breaks in the column. This tendency increased as the proportion of ether in the eluting solvent increased, but it was generally not serious provided the surrounding temperature was below 28°. The column temperature could be controlled by using jacketed columns, but this was not done in the present experiments and in our experience the chromatographic separations were not appreciably affected by variations in room temperature.

Analysis. The eluent from the columns was collected in 5 or 10 ml fractions, and the distribution of lipids was determined gravimetrically after the lipid residues from individual fractions had been transferred with a minimum of solvent to tared glass tubes. The position of individual reference compounds was determined by running a separate column for each compound. The distribution and recovery were also checked in some cases by the use of colorimetric methods. Cholesterol was measured by the method of Sperry and Webb (13). Glycerol was determined on hydrolysis products of the lipids by the method of Lambert and Neish (14) as modified by Korn (15). Phosphorus was measured by the method of King (16) after oxidation of the lipids with perchloric acid.

RESULTS

Large Florisil Column (30 g). The separation of a cholesterol ester, a triglyceride, free cholesterol, a diglyceride, and a monoglyceride is illustrated in Figure 1. The sequence of eluting solvents used in this chromatogram (Table 1) gave complete separation and nearly quantitative recovery of the five different compounds. The use of 10% methanol in ether as the final eluting solvent gave a sharp peak of monoglyceride, but also eluted extraneous material from the column as shown by a smaller peak following the monoglyceride. In subsequent work this was avoided by eluting the monoglyceride with 2% methanol in ether. Other experiments showed that much larger amounts (at least threefold) of the reference compounds could be separated without overlap on this column. It therefore seemed desirable to devise an elution procedure for a smaller column in order to achieve the same separation

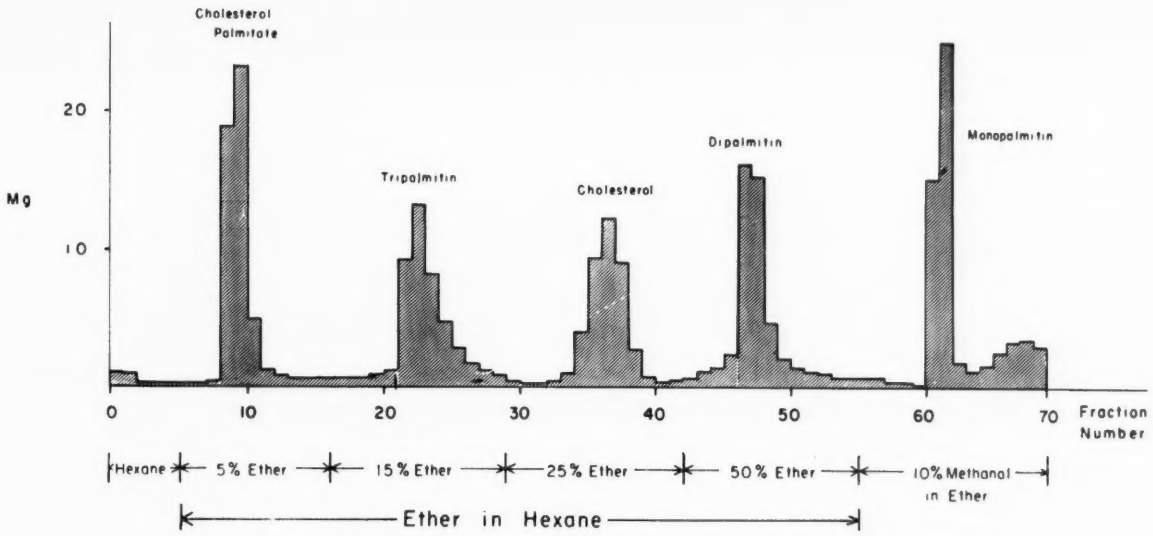

FIG. 1. Separation of lipid classes on 30 g column of Florisil. Ten ml fractions were collected. The column load consisted of 40 mg each of cholesterol palmitate, tripalmitin, cholesterol, 1,2-dipalmitin, and 1-monopalmitin.

with considerable saving in solvents and in the time involved.

Small Florisil Column (12 g). Chromatograms on this column were nearly all carried out with a new supply of Florisil which required deactivation with water for satisfactory separation of the reference compounds. Figures 2 and 3 illustrate the differences obtained when the Florisil was used with no added water, and with 7% added water. In the experiment shown in Figure 2, each of the compounds emerged much later from the chromatogram, the triglyceride overlapped the cholesterol, the diglyceride was only partially eluted, and the monoglyceride remained on the column. However, when the Florisil was hydrated with 7% water (Fig. 3), the chromatographic pattern was similar to that obtained with the large column (Fig. 1). In Figure 3 cholesterol and dipalmitin were both eluted with 25% ether in hexane. This gave a broader diglyceride peak than if the dipalmitin were eluted with 50% ether, but for practical purposes it was an advantage to eliminate the solvent change.

TABLE 1. ELUTION SCHEDULES FOR FLORISIL CHROMATOGRAMS

Eluent	30 g Column (2.0 cm × 17.0 cm)	Eluting Solvent	12 g Column (1.2 cm × 15.0 cm)
	ml		*ml*
Hydrocarbons	50	Hexane*	20
Cholesterol esters	120	5% ether in hexane	50
Triglycerides	150	15% ether in hexane	75
Cholesterol	150	25% ether in hexane	60†
Diglycerides	150	50% ether in hexane	60†
Monoglycerides	150	2% methanol in ether	75
Free fatty acids	150	4% acetic acid in ether	75

* Purified Skellysolve B.

† It may be found more convenient to elute both cholesterol and diglyceride fractions with 140 ml of 25% ether in hexane.

It may be noted that the degree of hydration of the Florisil had a greater effect on the position of triglycerides and diglycerides in the chromatogram than it did on the position of cholesterol. When the Florisil was hydrated with less than 6% water, the tripalmitin tended to trail into the cholesterol fraction. When it was hydrated with more than 6% water there was a tendency for the cholesterol and dipalmitin fractions to overlap. Hence, 7% hydration appeared to be an acceptable compromise, although the exact amount of water used was not critical.

Differences in degree of hydration of the Florisil also affected the separation of paraffin hydrocarbons and squalene. Both were eluted before cholesterol esters

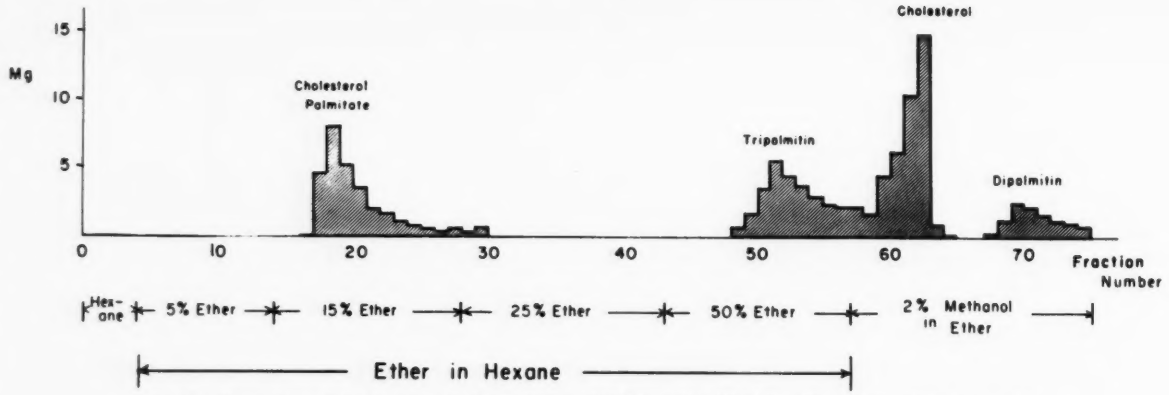

FIG. 2. Separation of lipid classes on 12 g column of highly active Florisil. Five ml fractions were collected. The column load consisted of 30 mg each of cholesterol palmitate, tripalmitin, and cholesterol, and 15 mg each of dipalmitin and monopalmitin. The amounts of eluting solvents were in some cases greater than those listed in Table 2.

on a column of Florisil hydrated with 7% water, but they were incompletely separated from one another (Fig. 4). However, on a column of Florisil with no added water the hydrocarbon and squalene fractions were completely separated (Fig. 5).

Free fatty acids were retained on the Florisil column during the elution with neutral solvents up to and including 2% methanol in ether, although in silicic-acid chromatography free fatty acids are eluted immediately after the triglyceride fraction (7). It seems likely that this difference is due to the basicity of Florisil, since palmitic acid was quantitatively eluted as a sharp peak from a Florisil column hydrated with 7% water by using 4% acetic acid in ether as the eluting solvent. The eluted material was very soluble in ether, and its melting point indicated that it was free palmitic acid rather than the magnesium salt. Small amounts of ether-insoluble material were also eluted with 4% acetic acid in ether, but whether this was magnesium acetate or some other substance was not determined.

In the results presented in Figures 1 to 5 the flow rate was controlled with a stopcock to 80 to 100 ml/hour, but in one experiment a mixture of cholesterol palmitate, tripalmitin, and cholesterol was eluted from a 12 g column at the maximum flow rate (160 to 180 ml/hour), and the separation of components was the same as that obtained at slower flow rates. Even at the controlled flow rate Florisil offers definite advantages over silicic acid in terms of saving time and material, as is indicated by the following figures. The preparation and elution of a 12 g column of silicic acid took, in our experience, approximately 24 hours and required about

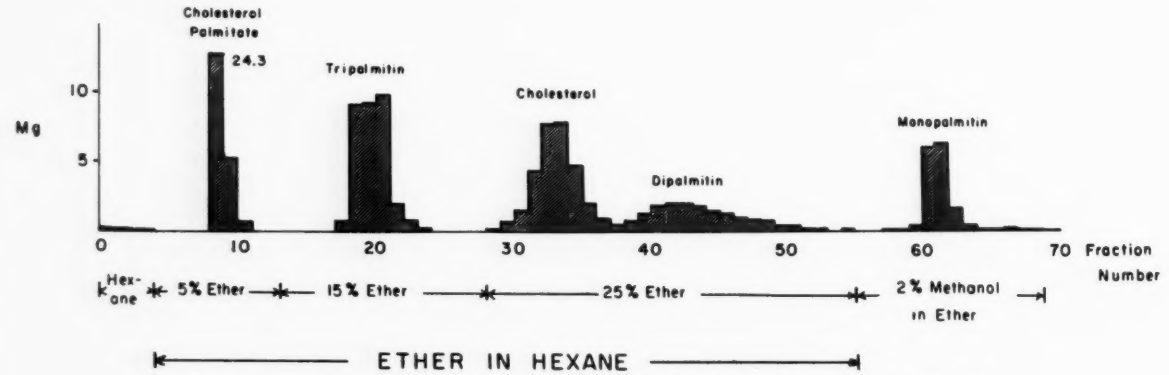

FIG. 3. Separation of lipid classes on 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected. The column load was the same as in Fig. 2.

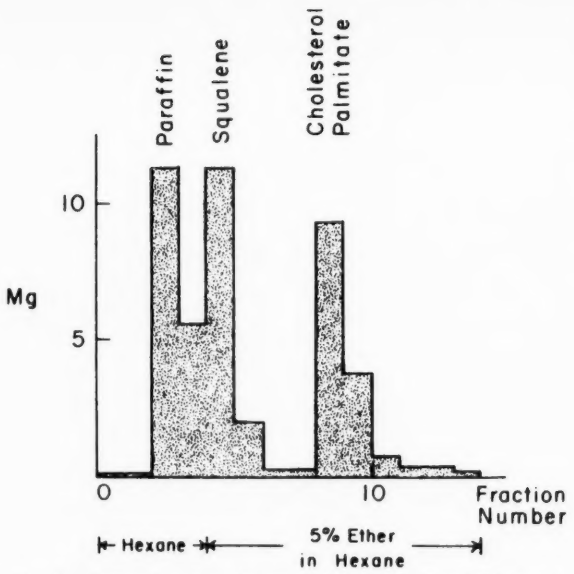

FIG. 4. Separation of a mixture of 15 mg each of paraffin, squalene, and cholesterol palmitate on a 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected.

1000 ml of solvent. A similar separation of neutral lipids was achieved on a 12 g column of Florisil in less than 4 hours with 350 ml of solvent.

The recovery of model compounds from Florisil columns was always greater than 90%, and in most cases was between 95% and 105%. Some of this variation was undoubtedly due to experimental errors in weighing.

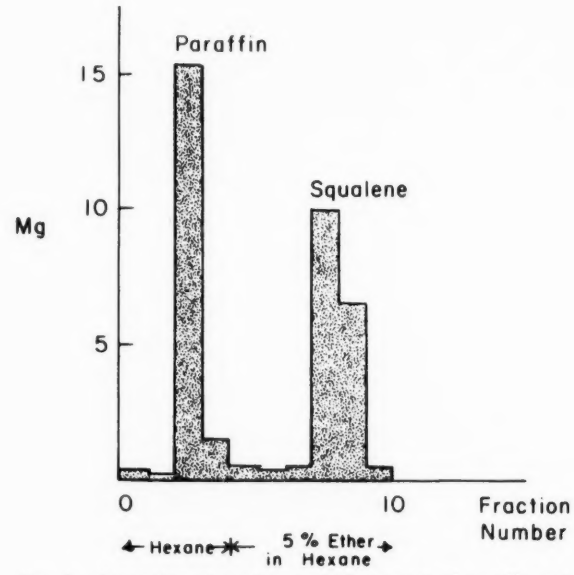

FIG. 5. Separation of a mixture of 15 mg each of paraffin and squalene on highly active Florisil. Five ml fractions were collected.

Separation of Naturally Occurring Lipid Mixtures. A number of chromatograms were run on 12 g Florisil columns with lipid extracts of normal rat liver (Fig. 6). Definite peaks corresponding to cholesterol ester, triglyceride, and free cholesterol fractions can be seen, and an analysis of these fractions for cholesterol and for glycerol indicated that the peaks actually contained the expected class of compound and that there was little or no overlapping of one class of compound with another. A similar chromatogram on rat blood lipids also gave major peaks corresponding to cholesterol esters, triglycerides, and free cholesterol (Fig. 7).

Phospholipids were not eluted with the solvents listed in Table 1. However, an attempt was made to separate the neutral lipids and phospholipids of rat liver by chromatography on a Florisil column under conditions similar to those used by Borgström with silicic acid (3). Five g of Florisil were added in portions to a column 1 cm in diameter filled with chloroform. A similar column containing 5 g of silicic acid and 2.5 g of Hyflo Super-Cel was prepared in chloroform as described by Hanahan *et al.* (17) and used as a control. Equal aliquots of a liver lipid extract dissolved in chloroform were applied to each of these columns. They were eluted first with 100 ml of chloroform and then with three 50 ml portions of methanol. As shown in Table 2, the phospholipid was recovered quantitatively in the first 50 ml of methanol from the silicic-acid column, while less than half of the total phospholipid was recovered from the Florisil column with 150 ml of methanol. The results shown in Table 2 suggested some fraction of phospholipids on the Florisil column, and further experiments were carried out in which smaller fractions were collected, but these indicated

TABLE 2. ELUTION OF LIVER PHOSPHOLIPIDS FROM SILICIC ACID AND FROM FLORISIL*

	5 g Silicic Acid 2.5 g Hyflo Super-Cel	5 g Florisil
Eluent *ml*	Phosphorus *μg*	Phosphorus *μg*
100 chloroform	8	4
50 methanol	1800	540
50 methanol	80	96
50 methanol	35	224

* Column load: 5 ml of chloroform containing approximately 55 mg of total lipid and 1800 μg of phosphorus.

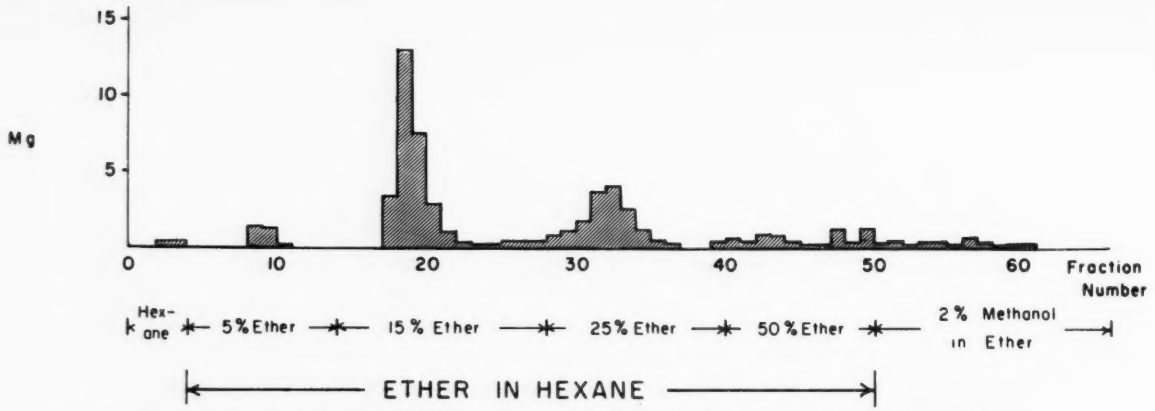

FIG. 6. Separation of rat liver lipids on 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected. The column load consisted of 245 mg of crude lipid extract.

that Florisil was probably not useful for the fractionation of phospholipids. Phosphorus was present in each of the small fractions and no sharp peaks were evident.

DISCUSSION

The results of this study showed that chromatography on Florisil could be used to separate classes of neutral lipids in much the same way as chromatography on silicic acid, and that use of Florisil rather than silicic acid offered a number of distinct advantages. Florisil columns were very simple to pack, the material required no prewashing, and the relatively coarse mesh used for these experiments permitted rapid flow rates. Florisil chromatography had the further advantage that free fatty acids were eluted after the various glyceride fractions, and this eliminated the possibility of their overlapping with triglycerides or diglycerides (5, 7).

The degree of hydration of the Florisil was shown to affect its chromatographic properties markedly, and, furthermore, its adsorption affinity for some types of lipids was affected more than that for others (Figs. 2 to 5). Hence, desired separations could be facilitated by using Florisil with the appropriate degree of hydration.

It seems probable that the highly active Florisil absorbs moisture from the atmosphere unless it is kept under anhydrous conditions. This may explain why the Florisil which was stored in the laboratory for several years differed in its adsorption properties from that obtained later. However, the original lot of Florisil did not alter noticeably during the period of months over which it was used, although the weather was very humid for part of the time and no special attempt was made to protect the Florisil from the atmosphere. This suggested that it may have reached an equilibrium condition in which it had little tendency to absorb more water. It therefore seems desirable either to store the

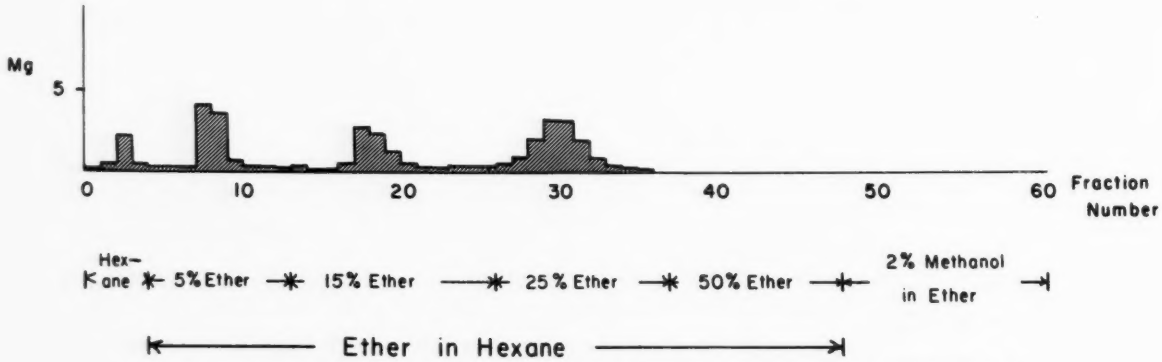

FIG. 7. Separation of rat blood lipids on 12 g column of Florisil deactivated with 7% water. Five ml fractions were collected. The column load consisted of 80 mg of crude lipid extract.

Florisil in the hydrated state or to maintain the highly active Florisil under anhydrous conditions in order to ensure consistent chromatographic results.

In this work the eluent from the chromatograms was collected in small fractions in order to determine the sharpness of the peaks and the degree of separation of individual compounds or groups of compounds. In our experience, the position of any given compound on the chromatogram was highly reproducible. Therefore, in studying naturally occurring lipid mixtures, it would probably be satisfactory to collect larger fractions in such a way that each fraction contained a major class of neutral lipid. It should not, however, be assumed that the position of a compound on the chromatogram is a guarantee that it belongs to a certain class of lipid. For example, it was found in other unpublished experiments that fatty alcohols were eluted together with triglycerides. It has been shown that the nature of the fatty acid moiety may affec. the position on silicic-acid chromatograms of lipids belonging to a given class (7), and this applies also to Florisil chromatograms (12). Therefore, positive identification of lipids eluted from Florisil can be made only on the basis of further characterization.

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Composition of aldehydes derived from some bovine lipids

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SUMMARY

The compositions of the aldehydes derived from phosphorus-free lipids of milk fat and ox heart, as well as from phosphatides of butter and ox heart, were investigated. In addition to normal aldehydes, considerable amounts of branched aldehydes were found, in which the branching occurred at carbon atoms situated α, β, and, in a few cases, γ to the terminal carbon.

In the literature a number of data can be found concerning the nature of the aldehydes derived from plasmalogens from various sources. Leupold (1) found octadecanal, hexadecanal, tetradecanal (probably), *cis*-octadecen-9-al and *cis*-octadecen-11-al released by plasmalogens from horse brain. The same aldehydes, with the exception of *cis*-octadecen-11-al, were obtained by Klenk and Friedrichs (2) from plasmalogens of horse carcass and heart muscle. The presence of octadecanal, hexadecanal, and an octadecenal in aldehydes liberated from horse brain has also been demonstrated by Kaufmann and Kirschnek (3). Gray (4) subjected the aldehydes from ox heart plasmalogens, in the form of their dimethyl acetals, to gas-liquid chromatography and was able to detect 18 different components, of which two could be identified as octadecanal and hexadecanal, respectively. In a recent publication Gray (5) describes the gas-liquid chromatographic analysis of the dimethyl acetals of aldehydes derived from ox-spleen choline plasmalogens. He based a tentative identification on the retention volumes of these acetals. In addition to normal aldehydes (dodecanal up to and including octadecanal), ten probably branched aldehydes were shown to be present. Finally, Van Duin (6) analyzed the aldehydes from butter plasmalogens in the form of their dinitrophenylhydrazones and established the presence of octadecanal and hexadecanal.

In the present work the aldehydes are liberated from the phosphorus-free lipids (7) of milk fat and ox heart. After conversion of the aldehydes, via the corresponding acids, into the methyl esters, the latter are separated by gas-liquid chromatography. This separation enables tentative identification of the fractions collected. The aldehydes generated from phosphatides of butter and ox heart are similarly analyzed and compared.

METHODS AND RESULTS

(*a*) *Aldehydes from Milk Fat.* Milk fat (1050 g) containing no detectable amounts of phosphorus (less than 0.0001% according to the method of Zilversmit and Davis [8]) was obtained from 4 liters of cream. In order to remove volatile aldehydes, if present, the fat was heated in 150 g portions, as described by De Bruyn and Schogt (9), for 5 hours at 10^{-6} mm and 120°. In model experiments it was ascertained that free aldehydes up to and including pentadecanal are removed. To liberate bound aldehydes, the fat was stirred at 50° for 4 hours with 4 liters of a mixture of glacial acetic acid and 2 N hydrochloric acid 1/1(v/v), after which the fat was washed free of acid and dried over magnesium sulfate. The liberated aldehydes were subsequently separated from the fat by degassing for 5 hours at 120° and 10^{-6} mm, and collected in a cold trap. The aldehydes were taken up in petroleum ether (b.p. 40°–60°), purified according to Van der Ven and de Jonge (10). To convert them into their 2,4-dinitrophenylhydrazones, the solution was brought in portions onto eight 15 × 270 mm Celite® 2,4-dinitrophenylhydrazine-HCl columns (11).

Because traces of short-chain carbonyl compounds from the solvent also give dinitrophenylhydrazones, the mixture was subjected to partition chromatography according to Kramer and van Duin (12) on eight 15 × 210 mm SiO_2 columns. The SiO_2 was prepared ac-

cording to Gordon *et al.* (13). Dinitrophenylhydrazones of aldehydes with a chain length of more than 11 carbon atoms (retention volumes less than that of undecanal) were collected, and the solvent removed under vacuum at 40°. To remove traces of nitromethane and improve the stability of the preparation, the dinitrophenylhydrazones were purified over two 15 × 170 mm alumina columns. The alumina (Imperial Chemical Industries) had previously been dried at 160° and deactivated with 8% water. Petroleum ether plus 5% diethyl ether was used as eluting solvent. Ninety-three mg of dinitrophenylhydrazones was obtained.

The aldehydes were regenerated by a modification of the method of Keeney (14): 47 mg of dinitrophenylhydrazones was heated under reflux with 45 ml of glacial acetic acid and 5 ml of a levulinic acid-water mixture 9/1 (v/v) for 30 minutes at 120°, and then for 15 minutes at 130°. The mixture was taken up in petroleum ether and washed with water. It was found that 6 mg of dinitrophenylhydrazones had not decomposed. The mixture was separated on a 10 × 170 mm alumina column. The aldehydes were eluted with petroleum ether containing 5% diethyl ether immediately before the undecomposed dinitrophenylhydrazones. After removing the solvent, the free aldehydes could be separated by gas-liquid chromatography. However, if it is desired to investigate the individual fractions further, difficulties arise due to partial oxidation of the aldehydes. Therefore, in all subsequent experiments, aldehydes were oxidized to acids and then converted into their methyl esters. The aldehydes liberated from 46 mg of dinitrophenylhydrazones were oxidized by the method of Mitchell and Smith (15). Freshly precipitated silver oxide was prepared from 1.4 g of silver nitrate and 8.3 ml of 1 N alkali, washed with water and then with dioxane, and transferred to a 25 ml Erlenmeyer flask with ground glass joint. To this were added the aldehydes in 9 ml dioxane and 2 ml water. The mixture was magnetically stirred and refluxed for 1 hour at 60° to 65°. After diluting with 50 ml of water, 4 ml of 1 N alkali was added and the excess silver oxide was filtered off. The alkaline filtrate was extracted with 25 ml of diethyl ether to remove any unoxidized aldehyde, and then acidified with 5 ml of 2 N H_2SO_4. Nineteen mg of acid was obtained,[1] which was taken up in a few milliliters of absolute diethyl ether and esterified with etherial diazomethane. The solution of esters was divided into two equal portions. Analysis of the first portion by gas-liquid chromatography is shown in Figure 1. The other half was dissolved in 3 ml of methanol and hydrogenated at room temperature, using 50 mg Adams' platinum oxide catalyst. The hydrogenated esters were also subjected to gas-liquid chromatography. Peaks *2*, *4*, *6*, and *8* correspond to the esters of normal tri-, tetra-, penta-, and hexadecanoic acids. Only peak *7* disappeared after hydrogenation and was therefore attributed to the methyl ester of a hexadecenoic acid. The increase after hydrogenation of peak *8* confirms this. As it is known that branched-chain methyl esters emerge from the column before the corresponding straight-chain esters (16), it was assumed that peaks *1*, *3*, and *5* were due to branched-chain esters.

Fractions representing peaks *3* and *5* were collected separately and saponified (steam bath, 15 minutes, 0.5 ml of 0.5 N methanolic KOH), yielding 2.7 mg of acid (m.p., 45°–47°) and 3.2 mg of acid (m.p., 20°–23°), respectively. To prove that these acids were branched, they were oxidized with chromic acid according to Archer and Hickinbottom (17). Parallel oxidations were carried out with the model branched-chain acids, while *n*-tetradecanoic acid (myristic acid) was run as a control. All the oxidations were carried out as described for the acid from the fraction in peak *3*. One and eight-tenths mg of this acid was mixed in a 10-ml flask with 0.2 ml of glacial acetic acid (Analar) and 0.1 ml of pure acetic anhydride. Fifty mg of powdered chromic acid was added to the cooled solution, which was subsequently stirred for 20 hours at 0°. Large, colorless crystals, presumably acetic acid, formed. After stirring for another 4 hours at room temperature, the reaction product was transferred with 12 ml of water into a 30-ml flask. The mixture was acidified with 2 ml of 0.1 N H_2SO_4 and sufficient dilute sodium bisulfite solution was added to decompose the unreacted chromic acid, just causing the color of the solution to change to green, taking care that no excess bisulfite

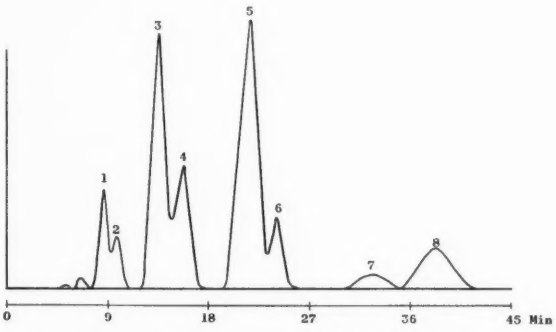

FIG. 1. Methyl esters corresponding to the aldehydes, obtained from milk fat. (Nitrogen flow 43 ml/minute, temperature 197°, pressure 38 cm Hg, Celite®/Apiezon L 4:1.)

[1] In a model experiment 12.7 mg of tetradecanal dinitrophenylhydrazone submitted to the same sequence of reactions yielded 4 8 mg of tetradecanoic acid.

was present. Subsequently, 12 ml of liquid was distilled off. The distillate was mixed with a solution of 0.06 g dinitrophenylhydrazine in 9 ml of 2 N hydrochloric acid. This reagent solution has previously been vigorously shaken with two 10-ml portions of petroleum ether to remove impurities. After allowing to stand for 16 hours at room temperature, the dinitrophenylhydrazones were extracted from the mixture with two portions of petroleum ether, and subsequently analyzed on the standard nitromethane column (12). Two bands, having retention volumes of 450 and 870 ml, respectively, were obtained. Band *1* was isolated, purified over an alumina column, and dissolved in 80 mg of chloroform. The ultraviolet absorption spectrum gave a maximum at 362 mμ, E = 0.444 cm²/mg. The retention volume and the ultraviolet maximum corresponded with those of acetone dinitrophenylhydrazone. From the extinction, 92 μg was present. Band *2* was probably acetaldehyde dinitrophenylhydrazone. This band was observed in all experiments, even in the oxidation of myristic acid, and might have originated from the solvents. The results of the oxidations are recorded in Table 1.

TABLE 1. OXIDATION OF BRANCHED ACIDS WITH CHROMIC ACID

Acids		μg Carbonyl Compounds Found After Oxidation			Yield of Theoretical
		Acetone	Butanone	Pentanone	
mg					*per cent*
2.0	11-Me-dodecanoic acid	195; 150	0	0	30
2.0	11-Me-tridecanoic acid	13	17	0	3
2.0	10-Me-tridecanoic acid	7	0	9	1
2.0	*n*-tetradecanoic acid	9; 9; 7	0	0	
1.8	acid from peak 3	92; 50	0	0	
1.7	acid from peak 5	29	14	0	

The acid from peak *3* gave only acetone, and in view of the retention time of its methyl ester, it is therefore 12-methyltridecanoic acid. Its melting point of 45°–47° is compatible with data from the literature: 53.3°, Weitkamp (18), 53.3°–53.6° Arosenius *et al.* (19).

The acid from peak *5*, which on oxidation gave acetone and butanone, is apparently a mixture consisting largely of 12-methyltetradecanoic acid. Its melting point of 20°–23° is in fair agreement with that of the optically active 12-methyltetradecanoic acid (m.p., 23.0) from lanolin (18). From the amount of acetone formed, a small percentage of 13-methyltetradecanoic acid must also be present.

From the results it follows that, apart from normal aldehydes, the aldehydogenic lipids in butterfat also yield 12-methyltridecanal and 12- and 13-methyltetradecanal.

(*b*) *The Aldehydes from Phosphorus-free Ox Heart Lipids.* From 12 kg of ox heart muscle, 92 g of phosphorus-free lipids was obtained, using the procedures previously described (7). The lipids were saponified, and the unsaponifiable fraction isolated by repeated extraction. Since the lipids had not previously been heated in high vacuum, the unsaponifiable fraction might contain free aldehydes in addition to the aldehydes bound as enol ethers (7). The unsaponifiable fraction was treated with silver oxide, and any acid formed was removed by alkali extraction. The aldehydogenic lipids are not affected by this treatment. The unsaponifiable fraction, free of aldehyde, was dissolved in 15 ml of petroleum ether and passed through three 33 × 270 mm Celite®-dinitrophenylhydrazine-hydrochloric acid columns (11), thus splitting off the aldehydes from α,β-unsaturated ethers and converting them into the dinitrophenylhydrazones. The latter were freed from sterols on an alumina column in which sterols are retained. Subsequently, the dinitrophenylhydrazones of aldehydes with more than 11 carbon atoms were isolated on the nitromethane column.

The purified dinitrophenylhydrazones, corresponding to about 58 mg of aldehydes, were investigated via the corresponding methyl esters as described under (*a*). In the gas-liquid chromatogram (Fig. 2) peaks *2*, *4*, *6*, *8*, and *10* correspond to the normal saturated esters (C_{14} to C_{18}). After hydrogenation peak *9* disappeared and was therefore probably due to the methyl ester of an octadecenoic acid. Fractions from peaks *1*, *3*, and *7* were collected separately and, after saponification, oxidized with chromic acid. The results are shown in Table 2. Of fraction *5*, insufficient material was obtained for an oxidation test.

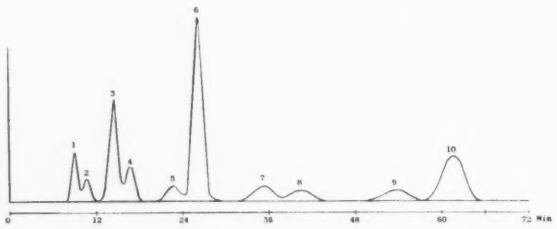

FIG. 2. Methyl esters corresponding to the aldehydes from non-phosphatide lipids in ox heart. (Nitrogen flow 30 ml/minute, temperature 197°, pressure 34 cm Hg, Celite®/Apiezon L 4:1.)

TABLE 2. CHROMIC ACID OXIDATION OF ACIDS OBTAINED BY OXIDATION OF ALDEHYDES OBTAINED FROM PHOSPHORUS-FREE OX HEART LIPIDS

Acid from Peak	μg Carbonyl Compounds Found After Oxidation		
	Acetone	Butanone	Pentanone
1	21	0	0
3	18	6	3*
7	16	3	0

* Paper chromatography of the dinitrophenylhydrazone indicated that this was probably pentanone-2.

In the mass spectrum of the acids from peaks *3* and *5*, the patterns of the hydrocarbon chains prove the acids to have a preference for splitting off C_3 and C_5 fragments. This behavior is to be expected for anteanteiso compounds[2] (CH_3—CH_2—CH_2—$C(CH_3)H$—), since splitting of the bonds on either side of a secondary carbon atom occurs easily. Experimentally, the validity of this determination of the position of branching was checked on a series of acids in the C_{14} group, including the anteiso- and anteanteiso isomers.

From these results it follows that, apart from the normal aldehydes C_{14} to C_{18}, the aldehydes derived from phosphorus-free ox heart lipids also contain 12-methyltridecanal, 11-, 12-, and 13-methyltetradecanal, 12-methylpentadecanal, and 14-, and possibly 15-methylhexadecanal.

(*c*) *The Aldehydes from the Phosphatides of Butter.* Two and seven-tenths g of phosphatide was isolated from 3 kg of butter, using the method of Koops (20). To liberate the aldehydes, the phosphatide, suspended in 25 ml of petroleum ether, was brought onto a 35 × 270 mm Celite®-dinitrophenylhydrazine-hydrochloric acid column. The dinitrophenylhydrazones (corresponding to 16 mg of hexadecanal) were purified and converted into methyl esters as described under (*a*). Because the amount of material was small, the total mixture was hydrogenated prior to gas-liquid chromatography (Fig. 3). Peaks *2*, *4*, *6*, *8*, and *9* correspond to the normal C_{14} to C_{18} esters. It is clear that branched chains are present in aldehydes obtained from butter phosphatides.

(*d*) *Aldehydes from Ox Heart Phosphatides.* As described (7), the phosphatides from ox heart were separated from the total lipids by means of dialysis, according to Van Beers *et al.* (21). Two g of this lipid mixture was investigated as described under (*c*). Three hundred and sixty mg of dinitrophenylhydrazones was obtained, corresponding to 206 mg of hexadecanal or to 613 mg of phosphatide, assuming choline as base and palmitic acid as fatty acid. The corresponding esters were subjected, before and after hydrogenation, to gas-liquid chromatography (Fig. 4). Only peak *9* (methyl ester of an octadecenoic acid) disappeared after hydrogenation. Peaks *2*, *4*, *6*, *8*, and *10* were in the positions of the normal fatty methyl esters C_{14} to C_{18}.

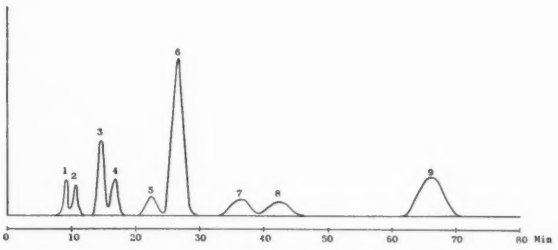

FIG. 3. Methyl esters corresponding to the aldehydes from butter phospholipids. Hydrogenated. (Nitrogen flow 100 ml/minute, temperature 200°, pressure 53 cm Hg, Celite®/Apiezon L 4:1.)

It might be expected that fractions representing peaks *1*, *3*, *5*, and *7* contained branched esters. This was confirmed for peaks *3* and *7* after saponification and oxidation with chromic acid (Table 3).

It may be concluded that peaks *3* and *7* contain both iso acids and anteiso acids. Thus the aldehydes derivable from ox heart phosphatides contain 13-methyltetradecanal, 12-methyltetradecanal, 15-methylhexadecanal, and 14-methylhexadecanal, in addition to normal chain compounds.

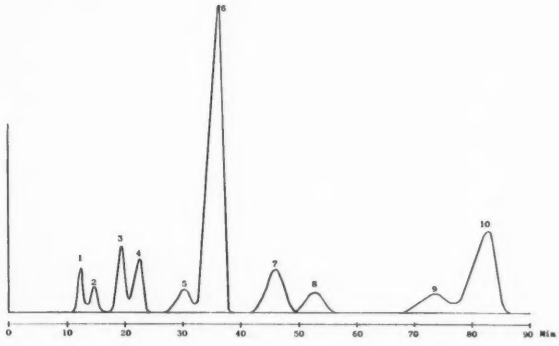

FIG. 4. Methyl esters corresponding to the aldehydes in ox heart phospholipids. (Nitrogen flow 30 ml/minute, temperature 197°, pressure 45 cm Hg, Celite®/Apiezon L 4:1.)

[2] The terms "anteiso-" and "anteanteiso-" mean that the branching occurs at carbon atoms situated β and γ to the terminal carbon, respectively.

TABLE 3. CHROMIC ACID OXIDATION OF ACIDS DERIVED FROM ALDEHYDES ISOLATED FROM OX HEART PHOSPHATIDES

2 mg Acid from Peak	μg Carbonyl Compounds Found After Oxidation		
	Acetone	Butanone	Pentanone
3	33	8	0
7	42	10	0

Synthesis of Model Substances. 11-Methyldodecanoic acid ethyl ester was prepared using the Kolbe synthesis, as described by Weedon (22) and Milburn and Truter (23), from ethyl hydrogen sebacate and 3-methylbutyric acid. The ester boiled over a range of 123°–135° at 3.5 mm. Yield based on sebacate, 51.4%. The free acid had a melting point of 40.6°–41.4°. Arosenius *et al.* (19) reported 40.6°–41.3°.

DL 11-Methyltridecanoic acid ethyl ester was prepared in a similar way from ethyl hydrogen sebacate and 3-methylpentanoic acid. The ester boiled at 153°–157° at 9 mm. Yield based on sebacate, 57%. The free acid melted at 14.5°–16.5°.

DL 10-Methyltridecanoic acid ethyl ester was prepared according to Cason and McLeod (24) by allowing propylmagnesium bromide to react with 10-keto-undecanoic acid ethyl ester (synthesized from dimethylcadmium and 9-carbethoxy-nonanoic acid chloride), dehydration of the 10-methyl 10-hydroxytridecanoic acid ethyl ester so formed and catalytic hydrogenation of the 10-methyltridecanoic acid ethyl ester. The yield of 10-methyltridecanoic acid ethyl ester (boiling range: 99°–106° at 3 mm) was 62% based on 10-keto-undecanoic acid ester.

The ethyl ester was converted via the free acid into the methyl ester. Gas-liquid chromatographic analysis showed that the final product was not pure. By fractionation over a preparative 1.5 × 200 cm gas-liquid chromatographic column (Celite® 150–300; 30% Silicone oil; 360 ml nitrogen/minute; temperature, 185°) the pure ester was obtained.

DISCUSSION

The complicated nature of the bound aldehydes from the various sources is bewildering at first sight. When we compare what is known about fatty acids, however, some analogies are clearly apparent. Shorland (25) demonstrated the presence of both iso acids and anteiso acids in various animal fats. The percentages of branched-chain acids are, according to Shorland, very small, in total never exceeding 1%. Table 4 records the composition of the aldehydes found in our experiments. It is surprising that the percentage of branched-chain compounds is much higher, viz., about 30%.

TABLE 4. PERCENTAGE COMPOSITION OF ALDEHYDES DERIVED FROM VARIOUS LIPID FRACTIONS UP TO AND INCLUDING C_{18}

Aldehydes	Butter-fat*	Non-phosphorus Ox Heart Lipids	Butter Phospha-tides	Ox Heart Phospha-tides
Branched C_{13}	4.5	—	—	—
n-C_{13}	3.5	—	—	—
Branched C_{14}	22.5	9	4.5	2.5
n-C_{14}	11	4	4	1.5
Branched C_{15}	39	19	13.5	5.5
n-C_{15}	7	4.5	6.5	5
Branched C_{16}		2	3.5	3.5
n-Hexadecenal	3.5		35	
n-C_{16}	8.5	36		46.5
Branched C_{17}		4.5	7	7.5
n-C_{17}		1.5	5	4
n-Octadecenal		4	21	4.5
n-C_{18}		15.5		19.5
Per cent branched		34	28	23

* The values in this column are too high. The aldehydes were, in this case, obtained by degassing at 120° from the fat after this had been treated with acid. During this procedure neither octadecanal nor hexadecanal was recovered quantitatively. The values given are of importance, therefore, only insofar as they reflect the ratio of the branched and nonbranched C_{13}, C_{14}, and C_{15} aldehydes.

According to Hartman (26), the branched acids can be related to the amino acids valine, leucine, and isoleucine. By decarboxylation and deamination of valine and leucine, branched C_4- and C_5-fragments are formed, which, on being built up with C_2-units, may give the even- and odd-numbered iso acids. Isoleucine could lead in the same way to the odd-numbered anteiso acids. This theory, when applied to the bound aldehydes, seems to fit fairly well.

The branched-chain aldehydes with an even number of carbon atoms were generally found to be of the iso type, while those containing an odd number of carbon atoms were apparently often a mixture of iso- and anteiso isomers. However, from the experiments with phosphorus-free ox heart lipids, it appeared that, among other aldehydes, 11-methyltetradecanal and 12-methylpentadecanal occur. These two aldehydes cannot be derived from amino acids in an analogous way.

An explanation for the facts observed can be given only when the role of the aldehydes in lipid metabolism is more clearly understood.

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The lipid residues in cytolipin H*

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SUMMARY

Analyses were done of the individual fatty acids in two preparations of cytolipin H and the lipid bases in one preparation. A number of fatty acids were present, lignoceric acid predominating. No 2-hydroxy fatty acids were present. Of the lipid bases, 93% was sphingosine and 7% was a more unsaturated residue (dehydrosphingosine?). No dihydrosphingosine was present. Analyses of a number of cytolipin preparations showed them to be similar to cerebrosides derived from nonnervous animal tissues in having a nonuniform distribution of normal saturated fatty acids: C_{24}, C_{22}, and C_{16} acids were present in high concentration, and C_{20}, C_{18}, and C_{14} acids were present in very low concentration.

On the basis of elementary and group analyses, cytolipin H, a lipid hapten isolated from human epidermoid carcinoma, was reported to contain fatty acid, a lipid base, glucose, and galactose in equimolar proportions (1). The carbohydrate residues were recently found to have the configuration of the disaccharide 4-O-β-D-galactopyranosyl-D-glucopyranose (lactose) (2). However, although evidence was presented indicating the presence of both fatty acid and a long-chain base, the lipid residues were not identified. A close correspondence of the carbon content of cytolipin H with that calculated for a molecule containing cerebronic acid and sphingosine led to the suggestion that these residues did indeed constitute the lipid portion of the cytolipin H molecule. It was subsequently found that 2-hydroxy fatty acids were absent. Scarcity of material prevented a conventional solution to this problem at the time, and micromethods had to be developed that would permit analysis of the fatty acid content of the original sample. A second specimen of cytolipin H was analyzed with respect to both its fatty acids and the long-chain base. These studies are presented in this report.

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METHODS AND MATERIALS

Cytolipin H was isolated from a human tumor (1). Analyses for this preparation and a second one isolated independently have been presented (1, 3). Fatty acid analysis was carried out, after hydrolysis and isolation, by paper chromatographic methods (4) and gas-liquid partition chromatography (5, 6). The analytical method for sphingosine and its derivatives was based on the formation of long-chain aldehydes followed by their separation using gas-column chromatography (6).

RESULTS

These studies were begun assuming that 2-hydroxy fatty acids were present in the cytolipin H molecule.

Fatty Acids in the Original Specimen. The material (13.7 mg) was hydrolyzed with 8 ml of 1.2 N HCl in 90% aqueous ethanol by heating under reflux for $2^1/_2$ hours. After neutralizing to pH 2.0, the ethanol was removed under a stream of nitrogen. Water (4 ml) was added and the acids and esters were extracted with five 10-ml portions of ether. After evaporating the ether, the residue was refluxed with aqueous 1.3 N NaOH. The hydrolyzate was brought to pH 2.0, extracted immediately with ethyl ether, and the ether was washed with water to avoid polymerization of hydroxy fatty acids. Evaporation of the solvent under nitrogen yielded 7.0 mg of fatty acids (fraction 1). A sample was taken for chromatographic analysis, and the re-

mainder (6.15 mg) was purified over the Mg^{++} salt (7), giving 2.3 mg of material (fraction 2) in which cerebronic acid, if present, would have been concentrated. Ninhydrin analysis (8) showed fraction 1 to contain about 10% sphingosine, whereas fraction 2 was free of sphingosine. The neutralization equivalent of fraction 2 was 373. Analysis of the fatty acids in both fractions by the paper chromatographic method is shown in Table 1 (preparation 1). Neither the total

TABLE 1. LIPID RESIDUES IN TWO PREPARATIONS OF CYTOLIPIN H

	Preparation 1		Preparation 2*	
	Paper Chromatography		Paper Chromatography	Gas-Liquid Chromatography
	Fract 1	Fract 2		
	per cent	*per cent*	*per cent*	*per cent*
Fatty acids				
Lignoceric (C_{24})	65.	96.	42.	46.
Behenic (C_{22})	5.	4.	16.	10.5
Arachidic (C_{20})	0.5			
Stearic (C_{18})	3.		tr.	1.
Oleic	15.			
Palmitic (C_{16})	11.		23.	27.
Myristic (C_{14}) (+ palmitoleic)	0.5		7.	0.5
Other			12.?†	15.5‡
Lipid bases				
Sphingosine				93.
Dihydrosphingosine				
Unknown (dehydrosphingosine?)				7.

* Hydrolytic conditions were different for the two methods; see text.
† Acids migrating more rapidly than myristic.
‡ C_{23}, 5.5%; unknowns between C_{22} and C_{24}, 10%.

fatty acid fraction nor the purified fraction contained any 2-hydroxy fatty acids; over 90% of the total fatty acid was composed of lignoceric (65%), oleic (15%), and palmitic (11%) acids.

Cupric Acetate Test. Prior knowledge of the absence of hydroxy fatty acids had been obtained by developing a quantitative modification of the cupric acetate test. It has been shown by Radin *et al.* (9)[1] that 2-hydroxy fatty acids form insoluble colorless chelates in ethanol with cupric acetate, whereas unsubstituted fatty acids give a blue color. We observed that all saturated acids (C_{14} to C_{24}) formed deep blue precipitates in methanol at 20°; unsaturated acids (oleic, linoleic) formed similar precipitates at −5°, as did 10-hydroxy and 12-hydroxy stearic acids. In contrast, 2-hydroxystearic and 2-hydroxylignoceric acids formed colorless or very pale blue precipitates at −5°. Solution of the precipitate and measurement of the blue color provided a quantitative assay. To a solution of 5 μmoles of fatty acid in 1 ml of methanol, 1.6 ml of 0.005 M cupric acetate in absolute methanol was added. After standing at 5° for 30 minutes, the precipitate was centrifuged at 5° and the supernatant solution removed. The precipitate was dissolved in 7.5 ml of *m*-xylene and the solution read against this solvent in a Coleman model 14 spectrophotometer at 680 mμ using a red filter. Results obtained with different mixtures of stearic and 2-hydroxystearic acids are shown in Figure 1*A*. A similar curve represents mixtures of lignoceric and cerebronic acids (Fig. 1*B*). Based on the neutralization equivalent, the purified fatty acid fraction of cytolipin H (preparation 1) gave a trace of color in excess of that obtained with a sample of lignoceric acid containing no cerebronic acid. This result indicated that no 2-hydroxy fatty acid was present.

Recalculation of Empirical Formula. The empirical formula for cerebronyl sphingosyl glucosidogalactoside is $C_{54}H_{103}O_{14}N$, for which the calculated percentages of C, H, and N are 65.5, 10.5, and 1.41, respectively. The percentages found for cytolipin H were 65.5, 10.3, and 1.38. An empirical formula calculated as a weight average of the fatty acids shown in Table 1 for preparation 1 is $C_{51.9}H_{98.4}O_{13}N$, for which the calculated percentages are C, 65.8; H, 10.5; and N, 1.44. The agreement between these values and those found for cytolipin H is still close, and thus the results based on elemental composition present a picture consistent with those based on analysis of larger structural units. The per cent hexose and iodine number for the revised empirical formula, 38.4 and 31.9, respectively, compare favorably with the values found, namely, 36.7 and 32.

Lipid Residues in a Second Preparation. A sample of 0.4 mg of a second preparation of cytolipin H was

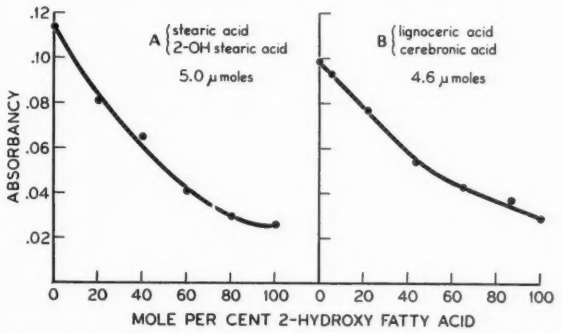

FIG. 1. Spectrophotometric detection of 2-hydroxy fatty acids in the presence of unsubstituted acids by the formation of colorless copper chelates.

[1] N. S. Radin. Personal communication.

hydrolyzed by refluxing for $2^1/_2$ hours with 4 ml of 1.2 N HCl in 90% ethanol. After adjusting the hydrolyzate to pH 2, evaporating the ethanol, and extracting with ether, the ether-extractable material was refluxed with dilute NaOH. The mixture was acidified to pH 2, extracted with ether, and the fatty acids were analyzed by paper chromatography. The results are shown in Table 1 (preparation 2).

For gas-liquid chromatographic analysis, 2.0 mg of cytolipin H was hydrolyzed for 6 hours at 100° with methanol-2 N HCl in a sealed tube. The lipid bases and fatty acid esters were separated and analyzed (6). The results of these analyses are shown in Table 1. Although the fatty acid analyses by the two methods were not identical, the results are in reasonably good agreement, especially in view of the difference in conditions of hydrolysis. Lignoceric acid was again the predominant fatty acid residue, although it was present in lesser amount than in the first preparation. Behenic and palmitic acids were present to a much greater extent in the second preparation. These three acids constituted 80% to 85% of the fatty acids in both preparations. About 10% of the fatty acids in the second preparation could not be identified with either of the analytical methods.

The lipid base is seen (Table 1) to be mainly sphingosine with a small quantity of an unknown base (presumably dehydrosphingosine). No dihydrosphingosine was present.

DISCUSSION

The results of this study show that the lipid residues in cytolipin H are neither a single fatty acid nor a single lipid base. Mixtures are obtained that may vary somewhat in composition, as is the case with many lipids isolated from natural sources. Whereas some of this variation may occur in the original tissue, much is attributable to selection that occurs during the process of purification. It is possible, however, to discern an interesting feature, namely, that the distribution of saturated fatty acids in cytolipin H is the same as it is in other glycolipids (from tissues apart from the nervous system). Thus in the two preparations of cytolipin H reported here, 80% to 85% of the fatty acid residues were composed of C_{24}, C_{22}, and C_{16} saturated acids. Only very small quantities of C_{20}, C_{18}, and C_{14} saturated acids were present. In a third preparation of cytolipin H, the combined percentage of C_{24}, C_{22}, and C_{16} acids was 95%. This may be compared with 93% for these three acids found, by Marinetti *et al.* (10), in a preparation of glucocerebroside from the spleen of a patient with Gaucher's disease, and 84% in a preparation of glucocerebroside from bovine spleen, by Skipski *et al.* (4). In two preparations of bovine spleen cytolipin, 91% and 86% of the fatty acids were found in this group. A summary of the results with these and another preparation is shown in Table 2. The reason

TABLE 2. FATTY ACID CONTENT OF CEREBROSIDES AND CYTOSIDES ISOLATED FROM NON-NERVOUS ANIMAL TISSUES

Preparation	Lignoceric	Behenic	Palmitic	Arachidic	Stearic	Myristic
Cytolipin H (prep. 1)*	65	5	11	0.5	3	0.5
Cytolipin H (prep. 2)†	44	13	25		1	0.5
Cytolipin H (prep. 3)*	62	10	23		5	
Bovine spleen cytolipin						
(prep. 1)†	28	19	48		2	1
(prep. 2)†	40	26	25	2	4	1
(prep. 3)*	65	10	25			
Bovine spleen glucocerebroside (4)†	46	27	11		4	
Gaucher spleen glucocerebroside (10)	46	27	20	3	3	

* Analysis by paper chromatography.

† Values are average of analyses by both paper and gas-liquid partition chromatography.

for the relative absence of C_{14}, C_{18}, and C_{20} acids from these two groups of sphingolipids is obscure, but may be related to the specificity of biosynthetic enzymes. This implicates a common pathway for the two types of substances (cerebroside and cytoside), and suggests that cytolipin may be synthesized by acylation of sphingosyllactoside (11).

When the carbon content of cerebronyl sphingosyl glucosidogalactoside ($C_{54}H_{103}O_{14}N$) was calculated, it was not appreciated that a similar result could be obtained for the behenic acid derivative ($C_{52}H_{99}O_{13}N$). The high result obtained in the determination of neutralization equivalent was particularly misleading in this regard, and the absence of 2-hydroxy acids from non-nervous animal tissues[2] stimulated us to develop methods for investigating this aspect of structure on the limited quantities of material at hand. These studies reinforce the statement (10) that older methods of single-value characterization of fatty acid content cannot be accepted without reservation, since a range of fatty acids is usually present in these compounds. The recent report (12) that the fatty acid in the glucocerebroside isolated from the spleen of a patient with Gaucher's disease was behenic acid very probably can be explained as the resultant of the fortuitous average of the right amount of C_{24} and C_{16} acids with a relatively smaller quantity of the C_{22} acid. Behenic acid has

[2] We are indebted to Professor E. Klenk for calling this fact to our attention.

not been found to predominate in any of the glycolipid preparations we have studied.

The lipid base in cytolipin H is almost entirely sphingosine. No dihydrosphingosine was present in the preparation studied, and a small quantity (7%) of an undetermined base (6), presumably an unsaturated derivative of sphingosine, was found. In comparison, two preparations of bovine spleen cytolipin were found to have 5% and 8% of dihydrosphingosine, the latter preparation also containing a small amount (3%) of the unknown base. The reported differences in degree of unsaturation between preparations of cytolipin H and bovine spleen cytolipin are thus found to be due to inconstant variations (from preparation to preparation) in the unsaturation of both fatty acid and lipid base residues. In view of the degree of variation in fatty acid composition indicated by the presence of oleic acid to the extent of 15% of the fatty acid in the first preparation of cytolipin H and its absence from the second preparation, the degree of unsaturation cannot be considered a reliable criterion of difference between human tumor and bovine spleen cytolipins (3).

Since the structure and configuration of the carbohydrate residues of cytolipin H have been shown to be that of the disaccharide lactose (2), the identification of the fatty acid and lipid base residues almost completes the structural identification. What must still be confirmed is that the glycosidic bond forms with the primary hydroxyl group of sphingosine (10), and what must still be determined is the configuration of this bond. This latter point of structure has still not been rigorously established for cerebrosides (13). The availability of antibody that reacts with cytolipin may make it possible to use the sensitive immunochemical method of hapten inhibition to provide answers to both questions when proper models are available.

We wish to thank Dr. Lewis Gidez for carrying out some of the early gas-liquid chromatographic analyses of fatty acids, and Mr. Stuart M. Arfin, Mr. Harvey Friedman, and Miss Kay Anthony[3] for capable technical assistance.

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The aerobic oxidation of unsaturated fatty acids and their esters: cobalt stearate-catalyzed oxidation of linoleic acid

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SUMMARY

The connection between metal catalysis and the initiation step in autoxidation is emphasized. The formation of free radicals occurs in reactions involving not only various valency states, but also oxygen-complexes of the catalyst. The kinetics of the initial phase of the metal-catalyzed aerobic oxidation of linoleic acid are examined in detail, and a number of mechanisms postulated. The rate constants for certain initiation reactions are evaluated together with the corresponding activation energies. The observed values are found to be in fair agreement with theoretical considerations and, in general, the evidence lends further support to the hypothesis that trace-metal catalysis and the initiation of autoxidation are intimately connected.

According to Bateman (1), the interaction of olefins with molecular oxygen is not only a subject of widespread industrial importance but it is also one of the most thoroughly understood chemical processes. However, the difficulties in the understanding of the initiation process were emphasized by one of us recently (2, 3) and are also reviewed in detail in a book (4). The propagation and termination processes of the chain reaction involved in the autoxidation of linoleic acid (5) are generally accepted as:

$$\left.\begin{array}{ll} \text{P(1)} & \mathrm{R\cdot} + \mathrm{O_2} \xrightarrow{k_o} \mathrm{RO_2^{\cdot}} \\ \text{P(2)} & \mathrm{RO_2^{\cdot}} + \mathrm{RH} \xrightarrow{k_p} \mathrm{ROOH} + \mathrm{R\cdot} \end{array}\right\} \text{(Propagation)}$$

$$\left.\begin{array}{ll} \text{T(1)} & \mathrm{RO_2^{\cdot}} + \mathrm{RO_2^{\cdot}} \xrightarrow{k_t} \\ \text{T(2)} & \mathrm{RO_2^{\cdot}} + \mathrm{R\cdot} \xrightarrow{k_{t'}} \\ \text{T(3)} & \mathrm{R\cdot} + \mathrm{R\cdot} \xrightarrow{k_{t''}} \end{array}\right\} \text{(Termination)}$$

Since P(1) is much faster than P(2), $[RO_2^{\cdot}] \gg [R\cdot]$ at oxygen saturation, the magnitude of which varies from olefin to olefin. In the case of linoleic acid, the partial pressure of oxygen in air is well above the level required for saturation; hence, as a result of $[RO_2^{\cdot}] \gg [R\cdot]$, the only significant termination reaction is T(1).

On the basis of stationary state kinetics this leads to the rate equation:

$$\text{R(1)} \quad \frac{-d[\mathrm{O_2}]}{dt} = \frac{d[\mathrm{ROOH}]}{dt} = \sqrt{r_i} \times \frac{k_p}{\sqrt{k_t}} \times [\mathrm{RH}],$$

where r_i is the rate of the initiation process leading to the formation of free radicals.

In the presence of peroxide, free radical formation would occur in unimolecular or bimolecular decomposition of the latter, but free radical production prior to peroxide formation has been attributed in most kinetic studies to

$$\text{I(1)} \qquad \mathrm{RH} + \mathrm{O_2} \rightarrow \mathrm{R\cdot} + \mathrm{HO_2^{\cdot}} \quad (5, 6).$$

If one accepts the value of ~ -35 kcal as ΔH for the reaction $H + O_2^{\cdot} \rightarrow HO_2^{\cdot}$, in accordance with Evans and Uri (7) and Uri (8), I(1) would be so endothermic that it could not contribute significantly to the initiation process.

Therefore the possibility of the participation of trace metals in the initiation process of autoxidation has been examined, and part of this study is concerned with the elucidation of the mechanism of the catalytic action produced by heavy metal compounds with fatty acids, porphines, and amino acids. Some preliminary results have been previously reported (2, 3). In this paper, which is self-contained as the first of a series, a detailed

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account of the mode of action of cobalt stearate is presented.

Some of the complex phenomena observed with cobalt catalysts were described by Bawn (9). In his investigations the importance of the cobaltic state is emphasized, and Bawn's general assumption is that the chain reaction of aerobic oxidation is initiated by an electron transfer reaction between cobaltic ion and either the substrate or its hydroperoxide.

EXPERIMENTAL

Materials Used. Linoleic acid was prepared from tobacco seed oil (rich in linoleic acid) by a procedure involving saponification, urea complex formation with fatty acids, fractional low temperature crystallization, and high vacuum fractionation. Five hundred g of tobacco seed oil (Younghusband, Barnes & Co., London) was dissolved in 500 ml of ethyl alcohol at 60°. Sodium hydroxide (108 g) was dissolved in 400 ml of water, allowed to cool, and added to the alcoholic solution. Saponification is indicated by a substantial rise in temperature and the appearance of a brown-red homogeneous solution. After cooling, aqueous H_2SO_4 (100 ml conc. H_2SO_4 + 400 ml H_2O) was added. The fat layer was separated and washed three times with distilled water. During this whole procedure oxygen-free *nitrogen* was passed through the liquid to prevent aerobic oxidation.

Urea (1.5 kg) was dissolved in 3 liters of boiling redistilled methyl alcohol. With nitrogen bubbling continuously through the liquid, the fatty acids obtained by saponification were added, and after cooling for about 2 hours under nitrogen, the mixture was left overnight at 0°. The precipitated urea complex was filtered under suction, and washed four times with methyl alcohol. Fatty acids were separated by adding the urea complex to 2.75 liters of 2 N hydrochloric acid at 60° to 70°, with nitrogen passing through the solution.

The crude linoleic acid was separated, dissolved in 2 liters of acetone, and left overnight at −35°. Saturated acids were then removed by filtering under suction through a No. 1 sintered glass funnel at −35°. After cooling to −50° and standing for at least 1 hour at this temperature, precipitated monoethenoid acids were removed by filtration at the same temperature. The solution was then kept overnight at −70° and the precipitated linoleic acid was separated by filtration through a Buchner funnel at −70°. Finally, the latter was washed with 100 to 200 ml of acetone cooled to the same temperature.

After removing traces of solvent under vacuum, the colorless, or pale yellow, fatty acid was distilled through a fractionating column under vacuum. The resulting colorless linoleic acid had the following properties: iodine value, 179; acid value, 199; peroxide value, 0.

Methyl linoleate (used sometimes for the purpose of comparison with linoleic acid) was prepared by dissolving 1 volume of linoleic acid in 5 volumes of redistilled methyl alcohol and passing anhydrous hydrogen chloride through the solution, which was cooled externally with ice, until two layers separated. After passing hydrogen chloride for an additional 5 minutes, the liquid was diluted with 10 volumes of distilled water and extracted three times with petroleum ether (b.p. 40°). The time required for esterification was about 15 to 20 minutes and the reaction was at least 99% complete. The combined extracts were washed with distilled water until they were free from acid, dried with anhydrous sodium sulfate, and the solvent evaporated under vacuum. The methyl linoleate was fractionated under vacuum, yielding a colorless product having the following properties: iodine value, 171; acid value, 0; peroxide value, 0.

Cobaltous stearate was prepared by treating 2 volumes of hot (90°) 0.25 M aqueous solution of sodium stearate with 1 volume of cold 0.375 M aqueous solution of cobalt chloride. The mixture was stirred at 70° to 80° for 20 minutes, filtered under suction through a No. 1 sintered glass funnel, and the precipitated cobaltous stearate was washed five times with small quantities of hot (80°) distilled water. The cobaltous stearate was dried in vacuum ($\leq$1 mm) for 2 hours at 75° to 80°.

Analysis: C, 64.5%; H, 11.2%; Co, 9.0%.
Calculated results from $(C_{17}H_{35}COO)_2Co \cdot 2H_2O$:
C, 65.4%; H, 11.2%; Co, 8.9%.

The solvent used for studying the oxidation reaction was either benzene (Analar, Hopkin and Williams) or cyclohexane (Howards) purified by shaking with acidified potassium permanganate, passage through a column of activated alumina (Spence, type O), distillation, and finally removal of impurities, particularly aromatic ones, by repeated adsorption on activated silica gel (B.D.H. 30 to 120 mesh) until the extinction measured at 220 mμ in a 1-cm cell against distilled water did not exceed 0.330. All other chemicals used were of Analar quality, B.D.H. or Hopkin and Williams.

For the purpose of tracer work, the following starting materials labeled with radioactive isotopes were obtained from the Radiochemical Centre, Amersham: cobalt chloride—Co^{60} (1.4 mc/mg), stearic acid —1-C^{14} (8.87 μc/mg), and linoleic acid —1-C^{14} (15.8 μc/mg). The linoleic acid was diluted approximately 100 times with carrier material before use. Co^{60}-labeled cobaltous stearate was prepared from labeled cobalt

chloride by the method described above, and after prior neutralization with sodium hydroxide, cobaltous stearate —1-C^{14} was prepared from labeled stearic acid by the same procedure.

Methods. In this investigation aerobic oxidation was studied in homogeneous systems, with benzene or cyclohexane as solvent. The solutions were introduced into test tubes (60 ml capacity) stoppered with ground-glass joints, and then shaken in a thermostatically-controlled bath with a specially built-in shaking device. Unless otherwise stated, experiments were carried out at 37° (±0.05°). Solvents were assumed to have reached equilibrium with air, and both the rate of shaking and the liquid surface area to volume ratio had no effect on the rate of reaction in its early stages. The latter factors become significant, however, if solvents are deaerated prior to their use, or if the reaction is followed to an advanced stage where attention must also be paid to the residual partial pressure of oxygen in the air phase.

Since in the early stages of the aerobic oxidation carbonyl formation is small, the analysis of the reaction mixture was concentrated on direct hydroperoxide determination and measurement of conjugated diene formation (in cyclohexane), allowing a correlation with hydroperoxide formed (further details are given under Results). The direct method of hydroperoxide determination is a modified iodometric standard method and is described by the authors elsewhere (10).

For the purpose of tracer work, Panax Counting Equipment 100C was used in conjunction with a G.E.C. EHM-2S thin end-window Geiger-Muller Counter built into a square lead castle. Counts were carried out in the plateau region of the counter (1500 to 1600 volts) with deadtime paralysis set to 300 μ sec. Thin layers of the material to be counted were produced on standard nickel planchettes by evaporation of the relevant solution with the aid of infrared rays emitted by a lamp mounted above the planchette. Corrections for self-adsorption of C^{14} were made by reference to a calibration curve obtained with cobalt stearate (1-C^{14}). For Co^{60} counts, an aluminium filter of density 900 mg/cm^2 was used to cut out β-rays.

RESULTS

Correlation Between Hydroperoxide Formation and Conjugation. Lundberg and Chipault (11) reviewed the early findings relating to the formation of conjugated hydroperoxides in diene oxidation. The discovery that there is a parallel occurrence of both hydroperoxide formation and conjugated diene formation was first made in the laboratories of the British Rubber Producers' Research Association during their fundamental studies of ethyl linoleate autoxidation (12). The literature discrepancies in the evaluation of a molar extinction coefficient of conjugated hydroperoxide derived from various linoleic acid esters are considerable and, to some extent, confusing. These apparently contradictory results, attributing to conjugated hydroperoxide a molar extinction coefficient in the range 22,000 to 32,000, are due to so many factors that their complete analysis is extremely intricate. The principal factors appear to be (*a*) lack of distinction between oxygen uptake and hydroperoxide formation, (*b*) insufficient knowledge concerning the stereoisomerism of conjugated hydroperoxide, and (*c*) the possible formation of a nonconjugated hydroperoxide or secondary reaction products in the relatively early stages of the oxidation.

In view of this complexity, correlation between diene conjugation and hydroperoxide formation is desirable in any special investigation, since it may be frequently affected by choice of experimental conditions; Table 1 presents the results of this correlation. The molar extinction coefficient of the linoleic acid hydroperoxide was evaluated at 232 mμ from the optical density of oxidizing linoleic acid in cyclohexane and the iodometric peroxide value, allowing for the absorption of unoxidized linoleic acid of nil peroxide value ($\epsilon = \sim 23$).

TABLE 1. MOLAR EXTINCTION COEFFICIENT OF LINOLEIC ACID HYDROPEROXIDE*

Method of Oxidation	Temperature	(232 mμ, cyclohexane)
Uncatalyzed	25°	28,600
Cobalt stearate catalyzed	25°	29,700
Uncatalyzed	37°	27,900
Cobalt stearate catalyzed	37°	27,300

* Ascertained at peroxide values not exceeding 150.

The main interest was concentrated on the early stages of oxidation, and there it was found that the correlation between iodometric peroxide value and absorbancy at 232 mμ, as a measure of conjugation, was unaffected by the presence of catalyst; moreover, it remained constant until about 5% of the linoleic acid present was converted into hydroperoxide. Above this level of oxidation there was an apparent progressive drop in the molar extinction coefficient of the hydroperoxide, both in the catalyzed and uncatalyzed oxidations, although this effect was more pronounced in the former.

THE COURSE OF LINOLEIC ACID OXIDATION

General Observations. Linoleic acid oxidation was first studied in the range of linoleic acid concentrations up to 0.2 M, and cobalt stearate concentrations of 4×10^{-5} M to 2×10^{-4} M in benzene and cyclohexane. Using the technique described in the section on experimental methods to follow the course of the reaction, it was shown that the relationship

$$\frac{d[\text{ROOH}]}{dt} \propto [\text{Linoleic Acid}]$$

(as illustrated in Fig. 1) was obeyed at least as far as a 5% conversion into hydroperoxide. At more advanced stages of oxidation there was a steady increase in the order of reaction rate with regard to linoleic acid, reaching the value of 1.5 at 10% to 15% conversion. The relationship

$$\frac{d[\text{ROOH}]}{dt} \propto [\text{Cobalt Stearate}]$$

(as illustrated in Fig. 2) was obeyed only up to about 1% of hydroperoxide formation. At a higher conversion to hydroperoxide, the order with regard to cobalt stearate decreased, reaching 0.5 at about 3% conversion.

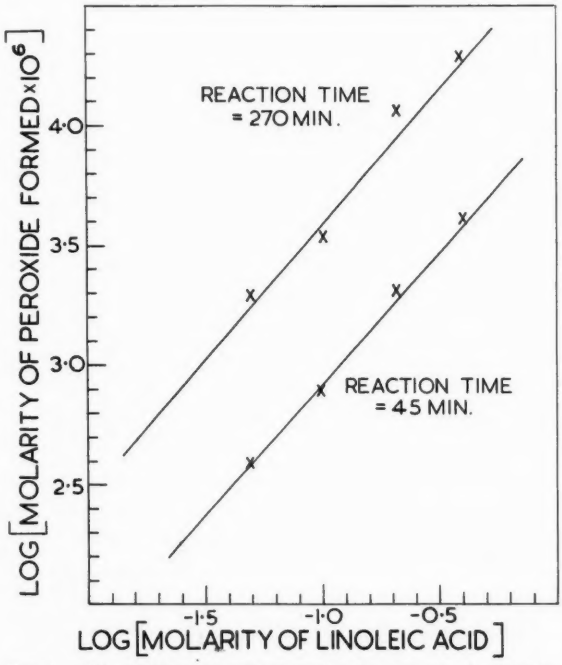

FIG. 1. Effect of linoleic acid concentration on rate of oxidation in cyclohexane at 37°. (Cobaltous stearate concentration = 4×10^{-5} M. Solution saturated with air.)

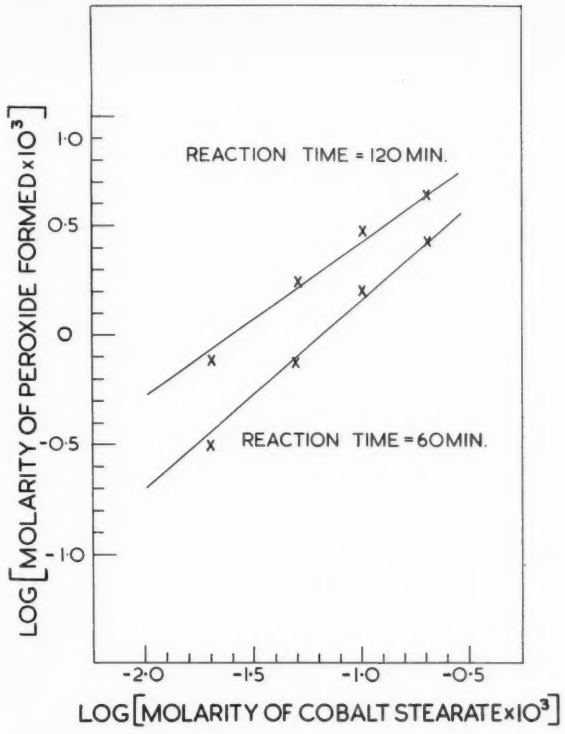

FIG. 2. Effect of cobaltous stearate concentration on rate of linoleic acid oxidation at 37°. (Linoleic acid concentration = 0.1 M. Solvent: benzene.)

Some unusual features were observed when the study was concentrated on the first 0.5% of oxidation at very low concentrations of cobalt stearate ($<4 \times 10^{-5}$ M). The prior equilibrium between solvent and air turned out to be so critical under these experimental conditions that no reliance was placed on the slow establishment of this equilibrium, but solvents containing the catalyst were aerated by bubbling about 10 liters of air through 250 ml of solution; an analogous technique was also employed with all nitrogen-oxygen mixtures used in these experiments. The basic anomaly is illustrated in Figure 3, in which the logarithm of the peroxide value after a fixed time interval (being thus a measure of rate) was plotted against the logarithm of cobalt stearate concentration in order to determine the order of reaction from its slope. The curve obtained was so irregular as to indicate that no simple reaction mechanism could be applied to its interpretation (this is dealt with in detail under Discussion). In view of these anomalous results, the experiment was repeated five times; although slight changes in the absolute value of the rate were observed, dependent on the absolute purity of the batch of linoleic acid employed, the basic phenomenon was found to be strictly reproducible and

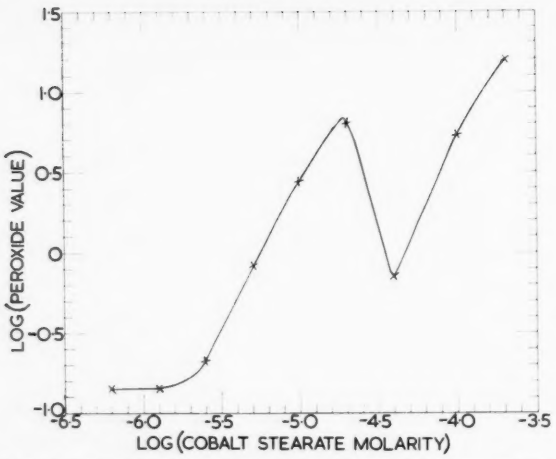

FIG. 3. Reaction order anomaly in the initial phase at low catalyst concentration. (Linoleic acid concentration = 0.1 M. Solvent: cyclohexane. Temperature: 37°. Time of reaction: 30 minutes.) Peroxide value = millimoles peroxide per g linoleic acid.

the maximum always occurred at the same cobalt stearate concentration. However, the critical concentration at which this maximum occurred was shown to be dependent on the temperature and the amount of oxygen in solution. This is illustrated in Figure 4,

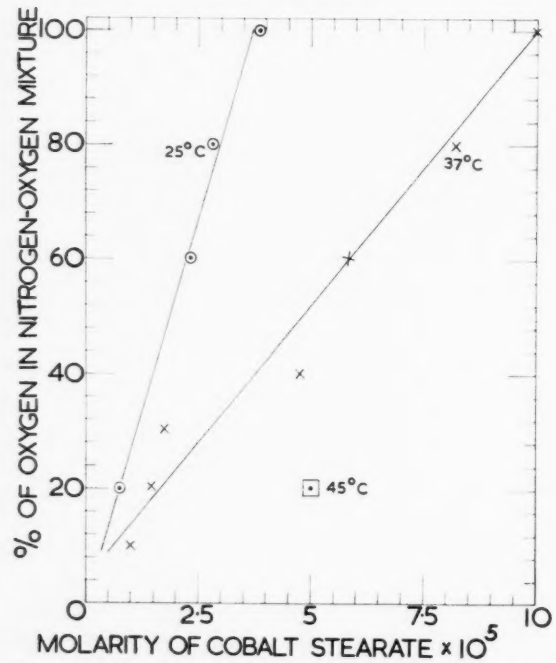

FIG. 4. Effect of temperature and oxygen content on catalyst concentration producing maximum initial rate of oxidation. (Linoleic acid concentration = 0.1 M. Solvent: cyclohexane.)

where the oxygen content was varied by saturating the solvent, in which catalyst had been dissolved, with nitrogen-oxygen mixtures of different composition, prior to linoleic acid addition.

At extremely low cobalt stearate concentrations (e.g., $\sim 2.5 \times 10^{-6}$ M), some departure from the normally observed first-order kinetics with regard to linoleic acid became apparent, but this point was not studied in detail.

Changes in the State of the Catalyst During Oxidation. Cobaltous stearate solutions in benzene or cyclohexane are very pale pink, but for practical purposes no color is discernible with 2×10^{-4} M solution in layers 2 cm deep. During oxidation the benzene solution assumes a readily observable yellow color in the range of 0.7% to 1% substrate oxidation. The stable yellow color was measured, and it was shown that the absorbancy of these solutions in the range 350 to 400 mμ was proportional to the original catalyst concentration. There was a steady increase in the absorption coefficient when the wave length was lowered in the above-mentioned region of the spectrum, but it was impossible to ascertain the full spectrum or even an ultraviolet peak because of interference by solvent and oxidized substrate.

When the reaction was carried out in cyclohexane solution, a similar yellow color developed in the same range of substrate oxidation, but this was unstable and its disappearance was accompanied by the formation of a pale yellow precipitate. In order to determine the composition of this precipitate, tracer studies were carried out with Co^{60} and C^{14}. When Co^{60}-labeled cobaltous stearate was used as catalyst, it was found that the precipitate contained 98.5% of the labeled cobalt, which must be regarded as quantitative precipitation within the limits of experimental error. In separate experiments carried out with cobaltous stearate (1-C^{14}) and linoleic acid (1-C^{14}), it was shown that the precipitate contained 0.2 atom of stearate-C^{14} and 2.8 atoms of linoleic acid-C^{14} per atom of cobalt.

Cobaltous-Hydroperoxide Reaction. At cobaltous stearate concentrations up to 10^{-6} M no catalytic effect was observed in the absence of detectable hydroperoxide; the measurements indicated a rate of oxidation which was indistinguishable from the apparently uncatalyzed autoxidation reaction in cyclohexane at linoleic acid concentrations of the order of 0.1 M. Under these conditions a catalytic effect can, however, be observed in the presence of hydroperoxide, and the rate law was established as

$$\frac{d[ROOH]}{dt} \propto [Co^{2+}]^{1/2} [ROOH]^{1/2} [RH]$$

(as illustrated in Figs. 5 and 6). The examination of

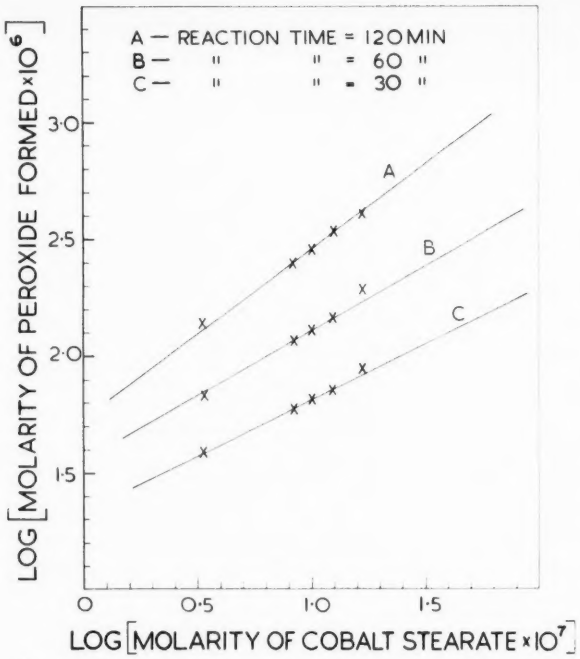

FIG. 5. Effect of cobaltous stearate concentration on the rate of the catalyst-hydroperoxide reaction at 37°. (Solvent: cyclohexane. Concentration of linoleic acid = 0.1 M, initially containing 2.5×10^{-4} M linoleic acid hydroperoxide.)

the temperature dependence of the rate between 25° and 45° in the above-mentioned range of experimental conditions revealed an over-all activation energy of 12.5 kcal (lines *A* and *B* in Fig. 7).

The Cobaltic-Linoleic Acid Reaction. Since the cobaltic catalyst formed in the course of the oxidation did not precipitate from benzene solution but attained a stationary concentration, it was possible to ascertain the kinetic law by variation of catalyst, substrate, and hydroperoxide concentration. It was found that the relationship

$$\frac{d[ROOH]}{dt} \propto [Co^{3+}]^{1/2}\,[RH]^{3/2}$$

(as illustrated in Figs. 8 and 9) was obeyed when the catalyst concentration was varied between 2×10^{-5} M and 2×10^{-4} M, and the substrate concentration between 0.05 M and 0.4 M. The rate was found to be independent of hydroperoxide concentration in the range 10^{-3} to 3.5×10^{-3} M (corresponding to a peroxide value range of about 30 to 120, with 0.1 M linoleic acid). Under these conditions the rate of the hydroperoxide-induced oxidation in the absence of metal catalyst was $\leq 2\%$ of the metal-catalyzed oxidation, and was neglected. The over-all activation energy for the cobaltic-catalyzed reaction was determined as 14 kcal (lines *C* and *D* in Fig. 7).

DISCUSSION

Apart from the intrinsic interest in connection with the oxidative rancidity of fats, there are several advantages in the use of linoleic acid and its esters in a study of autoxidation: (*a*) Relative ease of obtaining a pure preparation of a 1-4 diene. (*b*) In this structure the weakness of the α-methylenic C...H bond assures the predominant rupture of a single well-defined bond. (*c*) The chain reaction leads, for all practical purposes, in the initial stage to a single oxidation product, the hydroperoxide, which according to Hargrave and Morris (13) corresponds to 98% of the oxygen uptake. This feature does not apply to many other compounds where the multiplicity of products would make a kinetic study more difficult. (*d*) For all practical purposes the hydroperoxide initially formed is conjugated, and therefore the reaction can also be followed conveniently in certain solvents, such as cyclohexane and ethyl alcohol, by direct measurement of light absorption at 230 to 235 mμ, which leads to an increase in accuracy at low concentrations of hydroperoxide.

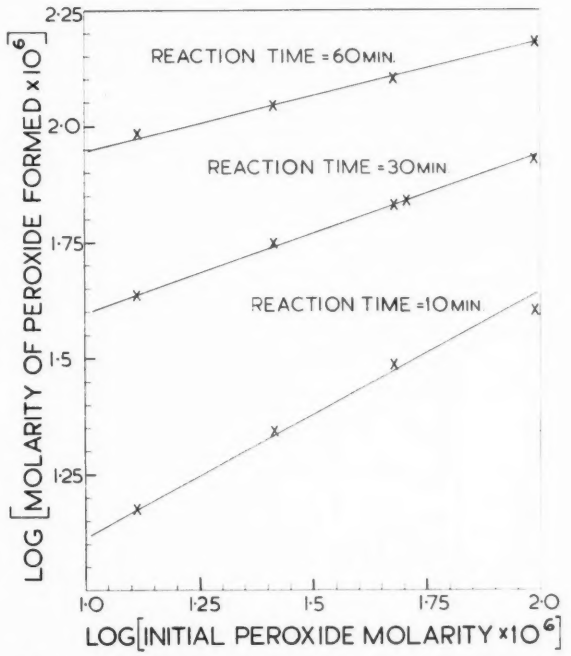

FIG. 6. Effect of linoleic acid hydroperoxide at low concentration on the cobaltous-catalyzed oxidation of linoleic acid at 37°. (Solvent: cyclohexane. Concentrations of linoleic acid and cobaltous stearate 0.1 M and 1.25×10^{-6} M, respectively.)

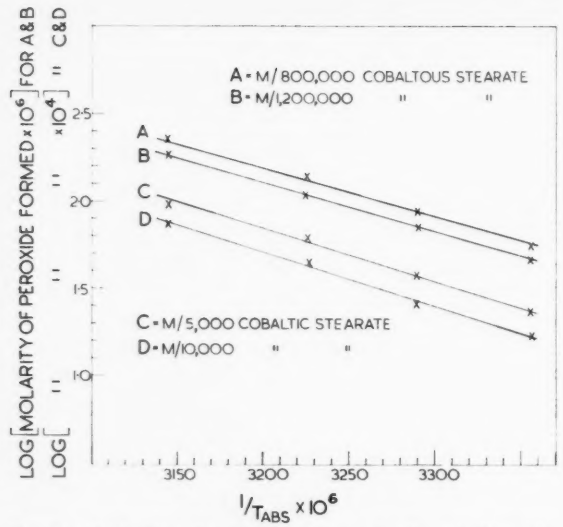

FIG. 7. Evaluation of activation energies for the cobaltous-peroxide (lines *A* and *B*) and cobaltic-linoleic acid (lines *C* and *D*) reactions. (*A* and *B:* linoleic acid concentration = 0.1 M, initially containing 2.5×10^{-4} M linoleic acid hydroperoxide. Solvent: cyclohexane. *C* and *D:* linoleic acid concentration = 0.214 M, initially containing 0.060 M linoleic acid hydroperoxide. Solvent: benzene.)

There are some problems relating to the isomerism of the peroxide which are not yet fully understood. These apply largely to the geometrical isomers of conjugated hydroperoxide formed during oxidation. Our quantitative correlation is in very good agreement with the value of $\epsilon = 28{,}000$ for the *cis-trans* isomer quoted by Sephton and Sutton (14). The predominant formation of *cis-trans* conjugated hydroperoxide from naturally occurring *cis-cis* linoleic acid would be in agreement with the rules postulated by Nichols *et al.* (15). Recently it was claimed that diperoxides are formed even in the early stages of autoxidation (16). However, it is noteworthy that analysis of the curve obtained by Privett and Nickell supports such diperoxide formation only at peroxide values of ≥ 150. For the purpose of our investigation this was considered an advanced stage, since we were almost exclusively interested in the first 1% of oxidation (corresponding to a peroxide value of ~30). The observed drop in the apparent molar extinction coefficient at a more advanced stage is in close agreement with the data of Privett and Nickell (16).

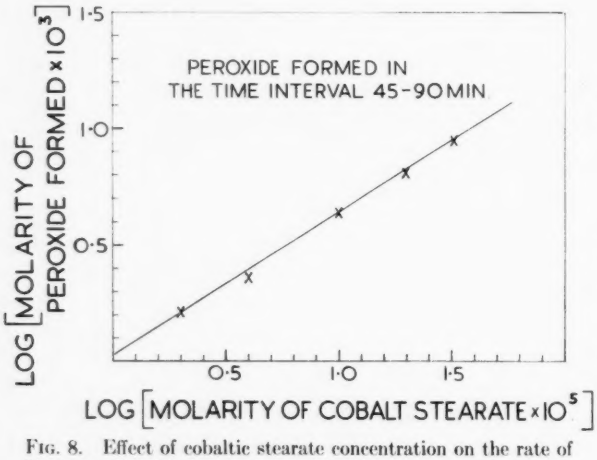

FIG. 8. Effect of cobaltic stearate concentration on the rate of linoleic acid oxidation in benzene in the presence of hydroperoxide at 37°. (Linoleic acid concentration = 0.214 M, initially containing 0.060 M linoleic acid hydroperoxide.)

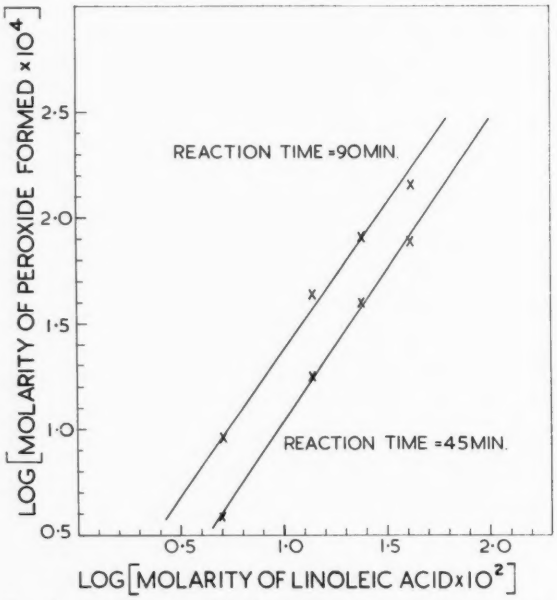

FIG. 9. Effect of linoleic acid concentration on the rate of its oxidation catalyzed by 10^{-4} M cobaltic stearate in the presence of excess peroxide at 37°. (Solvent: benzene. Initial hydroperoxide content of linoleic acid = 0.1 M.)

It is not unexpected that this drop is more marked in the catalyzed than in the uncatalyzed oxidation, since cobaltous stearate would not only be a catalyst for hydroperoxide formation but would also accelerate decomposition of the hydroperoxide and further reactions (e.g., polymerization, diperoxide formation) which are initiated by free radical intermediates of the cobaltous-hydroperoxide reaction. All these reactions lead to loss of conjugation in relation to peroxide content, thus producing the drop in the apparent molar extinction coefficient.

The main emphasis in this investigation was placed on the kinetic aspects of the initiation reaction rather than on the nature of the oxidation products. The initiation process turned out to be of very complex nature. Three distinctly different modes of initiation were ascertained.[1] (*a*) With the metal in the higher valency state the main reaction was found to be

$$\text{I(2)} \qquad Co^{3+} + RH \rightarrow Co^{2+} + R\cdot + H^{+}.$$

This is well substantiated by the kinetic observations, since $\sqrt{r_i}$ in the general rate equation R(1) is proportional to $[Co^{3+}]^{1/2}\ [RH]^{1/2}$. The over-all activation energy, ascertained as 14 kcal corresponds to

$$\frac{E_i}{2} + E_p - \frac{E_t}{2}.$$

Assuming an activation energy for propagation of 5 kcal (and neglecting E_t) (cf. reviews by Bateman [1] and Uri [4]) one arrives at a value of 18 kcal for the activation energy of the initiation reaction

$$Co^{3+} + RH \rightarrow Co^{2+} + R\cdot + H^{+}.$$

A calculation based on generally accepted data for k_p and k_t led to an estimate of 10^{10} (mole/liter)$^{-1}$ sec^{-1} for the temperature-independent factor of the rate constant. With such a high activation energy, this reaction is likely to be endothermic in benzene solution, and the assumption that the activation energy corresponds closely to the endothermicity for reactions of this type may be justified. In aqueous solution such a reaction would be very exothermic, if RH = linoleic acid (an estimate of ΔH amounts to -15 kcal). In benzene the cobaltic state (probably in some complex form) is much more stable in relation to the cobaltous state and, if indeed one is justified in using the term oxidation-reduction potential in nonaqueous solvents, the drop of the latter in benzene solution would exceed 1 volt.

It is noteworthy that the reaction

$$\text{I(3)} \qquad Co^{3+} + ROOH \rightarrow Co^{2+} + RO_2^{\cdot} + H^{+}$$

was not found to be significant, which is in contrast to a number of other observations in this field (9). Conceivably, the discrepancy between bond dissociation energy in R...H and in the corresponding hydroperoxide ROO...H is very much larger in the case of linoleic acid than in aldehydes or monounsaturated hydrocarbons. This would explain the predominance of the cobaltic-substrate reaction in the case of linoleic acid, and presumably also with other 1-4 dienes.

(*b*) The well-known reduction activation (17) is confirmed by experiments at such low cobaltous concentrations, where, in the absence of peroxide, no significant catalysis is observed. This initiation reaction is represented by

$$\text{I(4)} \qquad Co^{2+} + ROOH \rightarrow Co^{3+} + RO\cdot + OH^{-}.$$

The observed over-all activation energy of 12.5 kcal leads to an estimate of 15 kcal for the activation energy of the above-mentioned reaction. This is a much lower activation energy than that of the corresponding reaction with hydrogen peroxide in aqueous solution, the endothermicity of which would be ~35 kcal, but it is both qualitatively and semiqualitatively consistent with the observations made for the cobaltic-substrate reaction. The cobaltic state appears to be stabilized by comparison with normal aqueous systems to the extent of approximately 1 volt. A calculation of the temperature-independent factor of the rate constant for the cobaltous-peroxide reaction led to a value of 5×10^9 (mole/1)$^{-1}$ sec^{-1}, which is in fair agreement with observations in similar systems.

(*c*) With the metal in the lower valency state, the experimental evidence also indicates an initiation step which involves the cobaltous ion but no peroxide, and it has been shown that oxygen participates in this step. In an oversimplified manner it may be regarded as an electron transfer reaction of the type

$$\text{I(5)} \qquad Co^{2+} + O_2 \rightarrow Co^{3+} + O_2^{-}.$$

It was pointed out (2) that, in fact, this may be preceded by complex formation between the catalyst, substrate (in solvent), and oxygen according to the scheme:

$$\text{I(6)} \qquad Co^{2+}\cdot O_2(XH) + Co^{2+} \rightarrow Co^{2+} + Co^{3+}X^{-} + HO_2^{\cdot}.$$

Although it is possible to visualize monomolecular decomposition of the oxygen complex, experimental evidence favors a reaction of a higher order in this region. The anomalous curve in Figure 1, where the order of reaction at first rises with increasing cobaltous concentration, must be due to association of catalyst complexes. Molecular association of heavy metal soaps has been postulated recently (18). Perhaps the initiation reaction in this phase should be represented by:

$$\text{I(7)} \qquad [Co^{2+}(XH)]_n\cdot O_2 \rightarrow [Co^{2+}(XH)]_{n-1} + Co^{3+}X^{-} + HO_2^{\cdot}.$$

A quantitative interpretation of Figure 1 does not appear possible in view of the complexity of the system, but qualitatively the only possible interpretation would be based on the overlapping of at least three initiation reactions (one of them being negative or inhibitory).

[1] The use of ionic symbols does not necessarily imply the presence of ions as such, in simple form, but is merely an indication of the valency state of the metal. (This is the practice adopted by Bawn and his co-workers.)

It appears that positive initiation is produced mainly by reactions I(7), I(4), and I(2), and inhibition occurs via the reaction:

$$\text{I}'(1) \qquad Co^{2+} + RO_2^{\cdot} \rightarrow Co^{3+} + RO_2^{-}$$

which consumes free radicals. The reproducibility of the observations presented in Figure 3 merits at least a qualitative explanation. The rising curve on the right is explained readily as corresponding closely to cobaltic catalysis. The rising curve on the left is explained by free radical formation of increasing order due to association complexes, which is finally overtaken by an inhibitory reaction of the type I(1) involving a cobaltous association complex and $RO_2^{\cdot}$ radicals. It should be noted that the results in Figure 3 are obtained by sampling at a constant time interval and not at a constant amount of hydroperoxide formation, so that at higher cobalt concentrations the amount of peroxide is such that the cobaltic catalyst is predominant at the time of sampling.

The data in Figure 4 would be difficult, if not impossible, to explain unless the assumption is upheld that an oxygen complex, the formation of which is endothermic (to the extent of ~15 kcal), participates in the initiation reaction. This is important in the whole understanding of autoxidation, since it lends support to the hypothesis that the initial formation of hydroperoxide is due to the interaction between trace-metal impurities and molecular oxygen (3, 4). Once small quantities of peroxide have been formed the trace-metal reduction activation becomes important, and finally the higher valency state becomes effective as catalyst. The tracer experiments proved that a cobaltic linoleate compound was formed during the catalysis.

It would, in general, be an oversimplification to ascribe catalytic activity in autoxidation to one particular valency state, because it appears that a cyclic process which involves both the higher and the lower valency state occurs. This results in a certain stationary concentration of free radicals on which the propagation of the chain reaction depends. Among the heavy metal stearates, it was the cobaltous-cobaltic system which was found unequaled in its catalytic efficiency, but one must not generalize: e.g., among the porphine derivatives the ferrous-ferric system is the most active. Certain factors which influence the lability with regard to electron transfer or hydrogen transfer, and are not yet completely understood, appear to play a very important role in determining the catalytic efficiency of heavy metal catalysts which depends not only on the nature of the metal but also on its environment.

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Recombination with lipids of the lipid-free protein from canine serum (d 1.063-1.21, α_1) lipoprotein*

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SUMMARY

The protein (αP), prepared by delipidation of canine serum α_1-lipoprotein (αLP), when labeled with I^{131} and injected into dogs, was metabolized at the same rate as native αLP, labeled in the protein moiety with I^{131}. When αP-I^{131} was added to serum or injected into dogs, the radioactivity promptly appeared only in the αLP fraction, indicating a preferential interaction of the labeled protein with its own lipoprotein class. The nature of this interaction was not established. Mixing of αP-I^{131} with the low density lipoprotein class (βLP), in absence of serum, yielded two radioactive fractions, floating at d 1.063 and d 1.21. These two fractions had electrophoretic mobility similar to radioiodinated native βLP and αLP. In the absence of serum, αP-I^{131} reacted also with chylomicrons from serum or chyle. When the radioactive chylomicrons thus formed were injected into dogs, their disappearance from circulation paralleled that of an injected Lipomul (artificial triglyceride emulsion)-αLP-I^{131} complex. In both instances the disappearance of triglycerides was accompanied by appearance of radioactivity in the αLP fraction of plasma. When Lipomul was given intravenously to dogs injected with αLP-I^{131}, it combined with a small amount of this labeled lipoprotein. The possible participation of αLP in the metabolism of triglycerides is briefly discussed.

Studies from this laboratory have shown that the high density d 1.063–1.21 lipoprotein of human serum can be freed of practically all its lipids to yield a protein, apparently undenatured and identical from the immunochemical standpoint, with the native lipoprotein (1, 2). Further, *in vitro* experiments have shown that this lipid-free protein has high avidity for lipids and, under suitable conditions, combines with them to reconstitute the original protein-lipid complex (3).

The studies reported below deal with the *in vitro* and *in vivo* recombining capacity for lipids of the lipid-free protein from d 1.063–1.21 lipoprotein of canine serum.

MATERIAL AND METHODS

Sera from fasting normal dogs were pooled and chylomicrons removed by ultracentrifugation as previously described (4). The remainder, adjusted to d 1.063 by addition of solid NaCl (5), was centrifuged in a Spinco Model L Ultracentrifuge, 30.2 rotor, 79,420 × *g* at 16° for 18 hours. The top fraction (d 1.006–1.063 lipoproteins) was removed and the remaining serum brought to d 1.21 by addition of solid KBr (6). This serum was then spun in a Spinco 40 rotor at 105,000 × *g* at 16° for 24 hours and the top fraction (d 1.063–1.21 lipoprotein) removed.

Both d 1.006–1.063 and d 1.063–1.21 lipoproteins were purified by ultracentrifugation as reported previously (3) and dialyzed against 0.15 M NaCl. The preparations were considered satisfactory when neither albumin nor α_1-globulin was detected by starch gel electrophoresis (7, 8) and immunoelectrophoresis (9).

We will refer, for brevity, to d 1.006–1.063 and d 1.063–1.21 lipoproteins as β- and α_1-lipoproteins, respectively, according to their electrophoretic mobility.

Large aliquots of purified α_1-lipoprotein were freed of lipids by the ethanol-ether procedure of Scanu *et al.* (1) and the α_1-protein thus obtained was desiccated and stored at 0°. For use, an appropriate amount of this material was dissolved in 0.15 M NaCl.

I^{131} labeling of α_1-lipoprotein, α_1-protein, and β-lipoprotein was performed according to McFarlane (10); free iodine was removed by an ion exchange resin (Ioresin®, Abbott). The labeled protein was estimated

* This work was supported in part by grants from the United States Public Health Service and the Cleveland Area Heart Society.

to have about one atom of iodine per molecule. A molecular weight of 75,000 was assumed for the lipoprotein protein. The lipid moiety of the lipoproteins contained 4% to 5% of the total radioactivity.

Ultracentrifugal separation and purification of serum chylomicrons were performed as described previously (4), starting from pooled lipemic sera separated from dog's blood 6 hours after oral administration of 500 ml of 18% cream. Chyle chylomicrons were also used in our experiments. The chyle was obtained from two dogs in which the thoracic duct was cannulated as described by Albrink *et al.* (11) 3 hours following the administration of 500 ml of 18% cream. The separation of chyle chylomicrons was performed as for serum chylomicrons.

Total lipids were determined gravimetrically by Sperry's method (12). Total proteins were determined by Lowry's method (13). In lipemic samples, the protein content was also calculated by multiplying by the factor 6.25, the values of Kjeldahl nitrogen, obtained by the micromethod of Lang (14). In the *in vitro* studies αP-I^{131} [1] was mixed with whole serum, αLP, βLP, chylomicrons, or a cottonseed oil emulsion (Lipomul®, Upjohn). The mixtures were then incubated for 10 minutes at 24° and analyzed by ultracentrifugation and starch gel electrophoresis.

In the *in vivo* experiments six male mongrel dogs, 12 to 16 kg, were used. Throughout the course of the experiments they were kept in metabolism cages. Three days preceding the injection of the labeled lipoprotein and during the entire course of the experiments, 10 drops of Lugol's solution was added daily to the drinking water to prevent uptake of radioiodine by the thyroid. Each dog was given intravenously 1 to 2 mg[2] of αLP-I^{131} or αP-I^{131} of a specific activity of 20 to 30 μc/mg. Blood samples were taken immediately, 10, and 20 minutes after the injection of the labeled protein, and then at 24-hour intervals. Hematocrit values and αLP protein concentration were determined in all samples. αLP was separated by ultracentrifugation and its protein content determined by Lowry's method. In most of the serum samples the distribution of radioactivity was studied in the protein fractions separated by starch gel electrophoresis and in the lipoprotein fractions separated by ultracentrifugation. Starch gel electrophoresis analysis was performed according to Smithies (7), using a discontinuous system of buffers at pH 8.4; $\Gamma/2$, 0.05 (8). Amidoschwarz 10 B Bayer was used for protein staining and Spinco Oil Red O was used for lipid staining. Radioactivity was determined in a sodium iodide crystal scintillation detector (Tracerlab) with a counting efficiency of about 36%, determined by means of an I^{131} standard.

Calculations. The plasma volume was calculated by the usual isotopic dilution technique according to the formula $V_2 = C_1V_1/C_2$, where V_2 is the volume of circulating plasma in ml, C_1 the concentration of the total radioactivity of injected αLP-I^{131} or αP-I^{131} in cpm/ml, V_1 the volume of the injected material, and C_2 the radioactivity of dog's plasma in cpm/ml at 10 or 20 minutes from the injection. Half time, $(T/2)$ was calculated from the slope of the exponential line of disappearance of the radioiodinated lipoprotein protein from plasma. For the calculation of the volume of distribution, this line was extrapolated back to zero time. The curves of disappearance of the radioiodinated lipoprotein protein from plasma was obtained by plotting on a semilog paper time against per cent radioactivity retained, considering 100% the serum radioactivity at zero time. The value for total exchangeable pool size was the sum of the amounts of lipoprotein protein in the intra- and extravascular compartments. Turnover time (T_t) was calculated by the formula: $1.44 \times T/2$. The value for replacement rate was obtained by the formula: body pool in mg/g $\times$ fractional turnover time $(1/T_t)$.

RESULTS

Interaction of αP-I^{131} with Whole Serum, αLP, and βLP. αP-I^{131}, 100 μg, was mixed *in vitro* with 1 ml of fasting dog's serum and the mixtures were analyzed by ultracentrifugation at various salt densities. The data, summarized in Figure 1, indicate that αP-I^{131}, which alone sediments at d 1.21, floats at this density after in-

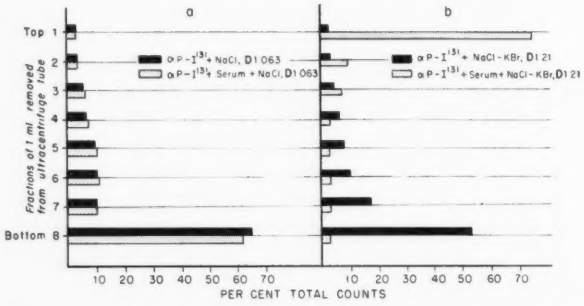

FIG. 1. Distribution of radioactivity in ultracentrifuge tubes after spinning αP-I^{131}, alone and mixed with serum, at d 1.063 (*a*) and 1.21 (*b*). Components: αP-I^{131}, 100 μg, specific activity 0.5 μc/mg, dog serum 8 ml, protein content 0.56 g. Densities were adjusted after incubation of the mixtures at 24° for 10 minutes. 30.2 rotor, 79,400 $\times$ *g*, 16°, 18 hours.

[1] Abbreviations used in the text: αLP = α_1-lipoprotein; αP = lipid-free α_1-protein; βLP = β-lipoprotein. αLP-I^{131}, αP-I^{131}, and βLP-I^{131} indicate radioiodinated αLP, αP, and βLP.

[2] In the text the weight of the lipoproteins is expressed in milligrams of lipoprotein protein.

cubation with serum. This radioactive top fraction at d 1.21 had electrophoretic and ultracentrifugal characteristics of a native αLP.

The ultracentrifugal analysis of a mixture of αP-I^{131} (100 μg) and αLP (1 mg) also showed presence of a radioactive fraction floating at d 1.21 (Fig. 2). Studies

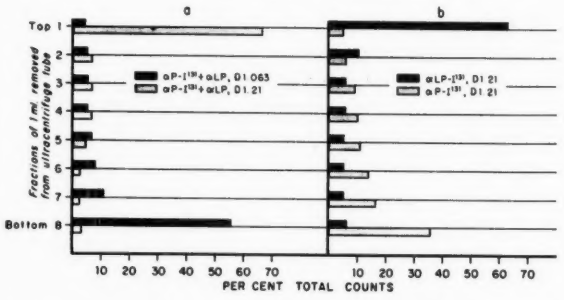

FIG. 2. Distribution of radioactivity after ultracentrifugation of a mixture of αP-I^{131} and αLP (*a*) at d 1.063 and 1.21. In (*b*) αP-I^{131} and αLP-I^{131} were spun alone at d 1.21. Components: αP-I^{131}, 100 μg, specific activity 0.5 μc/mg, αLP, protein content, 1 mg, αLP-I^{131}, 1 mg, specific activity 0.1 μc/mg. For experimental conditions see legend to Figure 1.

were also performed by starch gel electrophoresis. By this technique αLP gave three boundaries, each stainable for protein and lipids. αP also gave three boundaries, only stainable for protein, and with mobilities higher than the corresponding fractions of the native lipoprotein. The data, represented diagrammatically in Figure 3, indicate that αP-I^{131}, after incubation with αLP, has a reduced electrophoretic mobility and moves with αLP in the electrophoretic field.

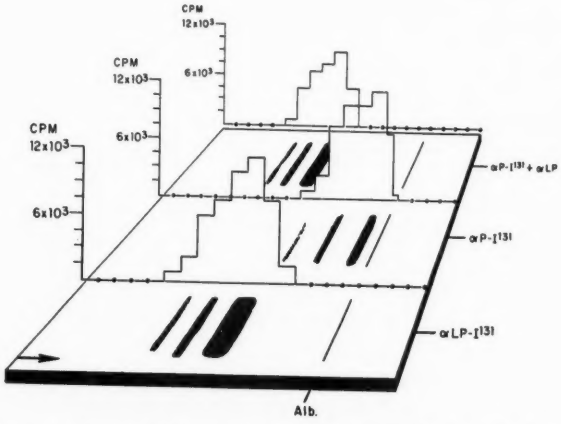

FIG. 3. Starch gel electrophoresis patterns of αLP-I^{131}, αP-I^{131}, and a mixture of αP-I^{131} and αLP. αLP-I^{131}, 2 mg, specific activity 50,000 cpm/mg. αP-I^{131}, 2 mg, specific activity 50,000 cpm/mg. The mixture contained 200 μg of αP-I^{131} and 2 mg of αLP. Staining: Amidoschwarz.

Mixtures of αP-I^{131} (100 μg) and βLP (1 mg), after ultracentrifugation at d 1.003, did not show a significant amount of radioactivity in the top layer. At d 1.063, the top fraction contained 33% of the total radioactivity (Fig. 4*a*). When this radioiodinated layer was removed and the remainder spun at d 1.21, 26% of the radioactivity contained in the ultracentrifuge tube floated (Fig. 4*b*). These labeled top fractions at d 1.063 and d 1.21, when respun at their respective densities, appeared in the top layer of the ultracentrifuge tube; when analyzed by starch gel electrophoresis, the distribution of radioactivity was the same as that of control samples of αLP-I^{131} and βLP-I^{131}.

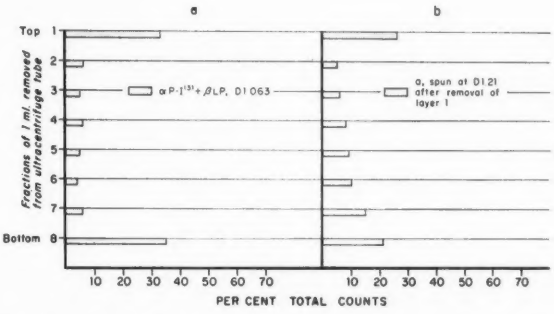

FIG. 4. Distribution of radioactivity in ultracentrifuge tubes after spinning a mixture of αP-I^{131} and βLP at d 1.063 (*a*) and at d 1.21 (*b*) after removal of the top fraction from (*a*). Components: αP-I^{131}, 100 μg, specific activity 0.5 μc/mg, βLP 1 mg. For experimental conditions see legend to Figure 1.

Addition of 1 mg of αLP to a mixture containing 100 μg of αP-I^{131} and 1 mg of βLP, prevented the interaction between αP and βLP.

When αLP-I^{131}, instead of αP-I^{131}, was mixed with βLP, the latter failed to become radioactive.

In the *in vivo* experiments, dogs were first injected with αLP-I^{131} and the rate of disappearance of the labeled protein from plasma was followed for 4 weeks. After a period of rest of 2 to 3 months, the same dogs were injected with αP-I^{131} and serum radioactivity followed again for 4 weeks. The results of a typical experiment are reported in Figure 5. Both αP-I^{131} and αLP-I^{131} had a monomial phase of metabolic degradation with a half time of 3.62 and 3.50 days, respectively. The values of half time, turnover time, and replacement rate (Table 1) for the injected αP-I^{131} and αLP-I^{131} were quite similar in all dogs studied.

αLP-I^{131} and αP-I^{131}, after intravenous administration into dogs, both migrated electrophoretically with the αLP fraction of serum (Fig. 6) and floated with the d 1.063–1.21 lipoprotein class.

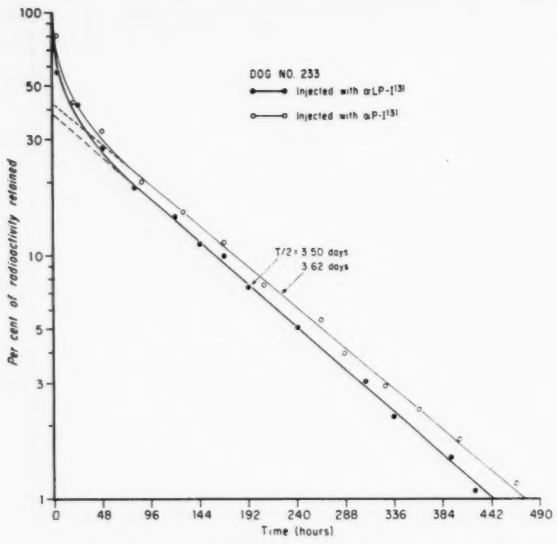

FIG. 5. Curves of disappearance of αP-I^{131} and αLP-I^{131} from circulation. Dose injected intravenously. αLP-I^{131}, 2 mg, specific activity 40 μc/mg; αP-I^{131}, 2 mg, specific activity 40 μc/mg.

Interaction of αP-I^{131} with Serum Chylomicrons, Lymph Chylomicrons, and an Artificial Fat Emulsion (Lipomul). Since fasting serum was used in the above experiments, the interaction of αP-I^{131} with chylomicrons could not be studied. Therefore *in vitro* experiments were first performed in which αP-I^{131} and αLP-I^{131} were mixed with both chyle and serum chylomicrons. These mixtures were spun at d 1.003 and the distribution of radioactivity determined. The mixtures of αP-I^{131} and chylomicrons from both chyle and serum showed a significant amount of radioactivity (36.6% and 34.2%) in the top 1 ml fraction (Table 2). This radioactive top fraction was not present when αP-I^{131} or αLP-I^{131} were spun alone at d 1.003 or when αLP-I^{131} was mixed with chylomicrons.

The problem was further investigated by studying the interactions of αP-I^{131} with Lipomul alone, or previously mixed with αLP. These results are summarized in Table 3. Formation of a labeled complex floating at d 1.003 occurred when αLP-I^{131} or αP-I^{131} were mixed with Lipomul alone. On the other hand, when Lipomul-αLP mixture was used, the labeled complex occurred after addition of αP-I^{131}, but not after addition of αLP-I^{131}. Lipomul-serum mixtures did not interact with either αLP-I^{131} or αP-I^{131}. In these experiments the mixtures of Lipomul-αLP and Lipomul-serum were purified according to the technique used for preparation of chylomicrons (10). As indicated in Table 3, lipemic sera from dogs in absorptive phase, when mixed with αP-I^{131} or αLP-I^{131}, failed to show a significant rise of radioactivity at d 1.003.

The following *in vivo* experiments, each one including two dogs, were performed. (*a*) Dogs were injected with either αP-I^{131} or αLP-I^{131}. A day later, when specific activity values for their plasma αLP was 5,000 to 10,000 cpm/mg, 500 ml of 18% cream was fed to the animals and 100 ml of blood withdrawn 6 hours later. The concentrated chylomicrons, separated and purified by ultracentrifugation at d 1.003 from the hyperlipemic sera, had a total lipid content of 20 mg/ml and contained no radioactivity. (*b*) Dogs were fed 500 ml of 18% cream and 6 hours later αP-I^{131} or αLP-I^{131} was

TABLE 1. TURNOVER OF αLP-I^{131} AND αP-I^{131} FOLLOWING INTRAVENOUS INJECTION INTO DOGS

Dog	Material Injected	Body Weight	P* Serum Conc.	Intravasc. Compartment Vol.	Intravasc. Compartment P	Extravasc. Compartment Vol.	Extravasc. Compartment P	Pool Size	T/2	Turnover Time	Replacement Rate
		kg	*mg/100 ml*	*ml*	*mg*	*ml*	*mg*	*mg*	*days*	*days*	*mg/g/day*
A	LP	14.9	293	590	1725	962	2820	4545	3.60	5.18	0.060
	P	15.0	284	538	1510	798	2266	3776	3.75	5.40	0.046
B	LP	13.0	273	495	1360	806	2200	3560	3.50	5.04	0.054
	P	13.2	262	470	1210	755	1970	3180	3.60	5.18	0.046
C	LP	16.0	248	676	1673	1057	2621	4294	3.51	5.05	0.053
	P	16.4	242	686	1660	1029	2490	4150	3.75	5.40	0.046
D	LP	13.2	272	520	1414	848	2306	3720	3.50	5.04	0.056
	P	13.4	278	538	1495	731	2032	3527	3.62	5.21	0.050
E	LP	12.6	281	510	1438	795	2223	3661	3.41	4.91	0.059
	P	13.1	282	500	1410	815	2298	3708	3.50	5.04	0.055
F	LP	12.0	260	480	1248	783	2035	3283	3.50	5.04	0.054
	P	12.6	278	486	1351	729	2026	3377	3.75	5.40	0.050

* P = αLP protein.

injected intravenously. Ten minutes later 100 ml of blood was drawn. The specific activity of this serum αLP was 5,000 to 12,000 cpm/mg. The chylomicrons, separated from these lipemic sera, had total lipid values of 25 mg/ml and no radioactivity. The results

TABLE 2. INTERACTIONS OF αP-I^{131} AND αLP-I^{131} WITH CHYLE AND SERUM CHYLOMICRONS*

Layers of 1 ml Removed from Top No.	αLP-I^{131}	αP-I^{131}	Chyle Chylomicrons Plus αLP-I^{131}	Serum Chylomicrons Plus αLP-I^{131}	Chyle Chylomicrons Plus αP-I^{131}	Serum Chylomicrons Plus αP-I^{131}
1+	12.0	12.3	14.7	14.0	36.6	34.2
2	12.1	12.1	12.0	12.0	10.4	9.0
3	12.1	11.7	12.1	12.1	9.2	9.1
4	12.2	11.4	11.9	11.8	8.2	9.4
5	11.8	11.9	12.0	11.8	7.8	9.4
6	12.4	12.2	12.1	12.1	8.5	9.2
7	12.3	11.7	12.0	12.3	8.8	9.0
8	14.1	16.7	13.2	13.9	10.5	10.7

* The data are expressed in per cent of total radioactivity present in each layer. Each value is the mean of the results from three experiments. 100 μg of αP-I^{131} or αLP-I^{131} with a specific activity of 0.5 μc/mg was used. Chyle and serum chylomicrons had a total lipid content of 50 mg. The mixtures containing the labeled lipoprotein protein and chylomicrons, following incubation for 10 minutes at 24°, were brought up to a volume of 8 ml with NaCl solution of d 1.003, then spun at 105,000 × *g* for 18 hours in a Spinco 30.2 rotor, 16°. Layers of 1 ml were removed by aspiration.

+ Top fraction.

TABLE 3. INTERACTIONS OF αP-I^{131} AND αLP-I^{131} WITH LIPOMUL ALONE OR MIXED WITH αLP AND SERUM*

Mixtures Analyzed			Results Radioactivity in Top Fraction at d 1.003
Fat Emulsion	Unlabeled Material Added	Radioactive Material Added	
			per cent
Lipomul	—	αLP-^{131}	73.2
Lipomul	—	αP-I^{131}	72.8
Lipomul	αLP	αLP-I^{131}	10.2
Lipomul	αLP	αP-I^{131}	28.2
Lipomul	Serum	αLP-I^{131}	10.6
Lipomul	Serum	αP-I^{131}	11.2
Lipemic serum	—	αLP-I^{131}	11.2
Lipemic serum	—	αP-I^{131}	10.9

* In all these experiments a constant amount of Lipomul containing 50 mg of total lipids was used. One mg of αLP or 1 ml of serum was incubated for 10 minutes at 24° with Lipomul. The mixtures were then equilibrated with 100 μg of αLP-I^{131} or αP-I^{131} and ultracentrifuged as indicated in the legend for Table 3. Lipemic sera were obtained from dogs 6 hours after feeding Lipomul.

obtained in (*a*) and (*b*) did not change when 500 ml of Lipomul instead of cream was fed to animals. To make it palatable, Lipomul was flavored with vanilla.

(*c*) Dogs were injected with either αP-I^{131} or αLP-I^{131}, and a day later, when specific activity values for serum αLP were the same as in the above experiments, 40 ml of Lipomul were injected intravenously and blood taken 10 minutes later. The particles of fat emulsion, separated by ultracentrifugation at d 1.003, contained a significant amount of radioactivity. Their content of labeled protein which had a specific activity of 4,000 to 10,000 cpm/mg was 200 μg/100 mg of total lipids.

(*d*) Dogs were given 40 ml of Lipomul intravenously, and 10 minutes later αP-I^{131} or αLP-I^{131} was injected intravenously and blood withdrawn. The chylomicrons contained about 500 μg of labeled protein/100 mg of total lipids. The specific activity of this protein was approximately of the same order of the soluble αLP in plasma (8,000 to 12,000 cpm/mg).

Mode of Disappearance from Plasma of I^{131} Chylomicrons and Lipomul αLP-I^{131} Complex. For these

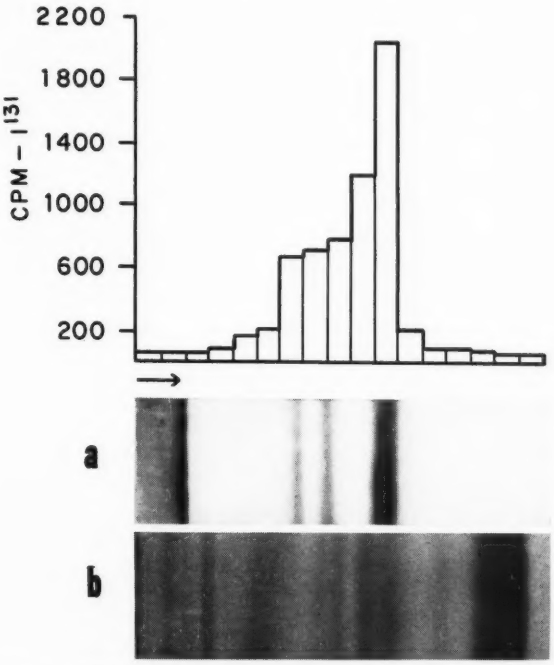

FIG. 6. Starch gel electrophoresis pattern of a serum from a dog injected with αP-I^{131}. The starch block was divided into three slices by two cuts parallel to the plane of migration. Upper curve: distribution of radioactivity in each one-half cm segment of starch; (*a*) pattern stained with Oil Red O; the broad boundary close to the origin is βLP, the other three boundaries correspond to αLP; (*b*) pattern stained with Amidoschwarz. Arrow indicates direction of migration.

experiments a large amount of chylomicrons separated from dog lipemic sera was used. Labeling of the chylomicron protein was performed according to the following technique: chylomicrons were equilibrated for 10 minutes at 24° with αP-I^{131}. About 100 μg of αP-I^{131} was added for each milligram of chylomicron protein. The mixture was then diluted with 0.15 M NaCl and spun at 9,000 × g for 10 minutes in a Spinco Model L ultracentrifuge, rotor 30.2, 16°. The top fraction obtained was layered under 0.15 M NaCl and centrifuged for an additional 18 hours at 105,000 × g in a Spinco 40 rotor. The chylomicron fraction, which floated at the top of the tube, had specific activity values up to 5 μc/mg of chylomicron protein.

The labeled chylomicrons were injected intravenously into two dogs and the rate of disappearance from plasma followed by determining chylomicron radioactivity and serum absorbancy values at 660 mμ of blood samples taken at 2-minute intervals. Fall of chylomicron radioactivity paralleled the fall in absorbancy at 660 mμ of whole serum. The half time of disappearance of chylomicrons from blood was about 10 minutes. As indicated in Figure 7, the fall of radioactivity of serum chylomicrons was accompanied by rapid appearance of

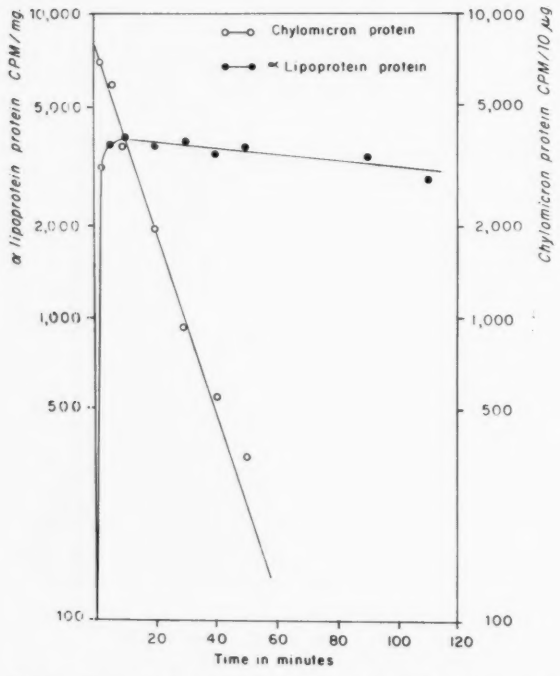

FIG. 7. Curve of disappearance from circulation of chylomicrons, labeled in the protein moiety with I^{131}. Specific activity values are expressed in cpm/mg of αLP protein and cpm/10 μg of chylomicron protein.

radioactivity in plasma αLP. No significant radioactivity appeared in plasma βLP.

Two more dogs were given Lipomul-αLP-I^{131} complex intravenously. This complex was prepared by mixing 50 to 60 ml of Lipomul with αLP-I^{131} of a specific activity of 40 μc/mg. Radioiodinated lipoprotein (100 μg) was added to each milliliter of Lipomul. After incubation for 10 minutes at 24°, the mixture was treated in the same manner as for the purification of labeled chylomicrons. When the complex Lipomul-αLP-I^{131} thus obtained was injected into dogs, its rate of disappearance from plasma was similar to the one observed with labeled chylomicrons. Also, in this case the plasma αLP became radioactive promptly.

DISCUSSION

These studies have shown that the protein (αP), prepared by delipidation of dog serum α_1-lipoprotein (αLP), when labeled with I^{131} and injected into dogs, is metabolized at the same rate as the radioiodinated protein moiety of a native αLP. The results, together with previously reported physicochemical (1) and immunochemical findings (2), strongly suggest that the αLP protein is not significantly changed by removal of lipids by the ethanol-ether procedure at −20° employed (1).

The present studies have also shown that when αP labeled with I^{131} is added to fasting or lipemic serum or is injected into dogs in absorptive or postabsorptive phase, radioactivity promptly appears only in the serum lipoproteins of density class d 1.063–1.21 having electrophoretic mobility of αLP. This indicates that the labeled αP interacts readily with its own class of high density lipoproteins, without exchanging with the protein moiety of low density lipoproteins (βLP) and chylomicrons. Similar findings were obtained by Gitlin *et al.* (15) after injection into humans of αLP, labeled in its protein moiety with I^{131}.

The exact nature of the interaction occurring between αP and αLP cannot be defined on the basis of our findings. It could be a simple process of adsorption of αP to αLP with formation of a complex with electrophoretic and ultracentrifugal properties similar to native αLP. Another possibility is that an actual transfer of a small amount of lipid occurs from αLP to αP, a transfer which may give a full complement of lipid to αP (which becomes αLP) at the expense of the donor native αLP. A third possibility is that of an exchange reaction between αLP and αP. In previous studies (3), samples of human serum αLP were equilibrated with various amounts of human serum αP-I^{131} and the mixtures spun at d 1.21. The distribution of radioac-

tivity between top and bottom fractions varied in a linear fashion with the mole fractions of the components. These results, and the fact that the radioactive top fractions had the electrophoretic and ultracentrifugal properties of a native human αLP, were interpreted as evidence for an exchange reaction between αP-I^{131} and αLP. Whether those findings can be applied to canine serum αP remains to be established.

It has been reported, and our *in vitro* and *in vivo* data are in agreement, that no exchange occurs between the protein moieties of αLP and βLP (15, 16). An exchange of lipids, to isotopic equilibrium, however, takes place involving cholesterol (17) and phospholipids (18, 19). Our present studies have shown that when αP-I^{131} is mixed with βLP, in absence of αLP, the mixture analyzed by ultracentrifugation at d 1.21, shows a radioactive top layer, which migrates electrophoretically in the αLP zone. These results, which are similar to the ones obtained with mixtures of human αP-I^{131} and βLP (3), indicate that either an actual transfer of lipids occurs from βLP to αP, to provide αP with enough lipid to re-form a lipoprotein complex similar to a native αLP$_1$ or an αP-I^{131}-βLP complex forms with a density between that of βLP and that of αP-I^{131}. Some reservations must be made until chemical and immunochemical characterization of the radioactive fraction floating at d 1.21 has been performed.

We have already mentioned that αP-I^{131}, when mixed with lipemic serum or plasma, fails to interact with the chylomicron fraction. Yet, when αP-I^{131} is mixed with purified samples of chylomicrons, in absence of serum, a radioactive top layer appears after ultracentrifugation of the mixture at d 1.003. It seems, therefore, that serum prevents the interaction between αP-I^{131} and chylomicrons. This is probably related to the fact that αP-I^{131} interacts preferentially with the αLP of serum to yield a product, αLP-I^{131}, which, as shown by our findings *in vitro* and *in vivo*, does not interact with chylomicrons.

It has been reported that αLP is one of the components of the chylomicron protein (4, 20). In view of the preferential interaction which occurs between αP and αLP, the question arises as to whether the αLP of chylomicrons is involved in the interaction chylomicron-αP-I^{131}. If this could be proved, it may furnish a basis for labeling specifically, *in vitro*, the αLP moiety of chylomicrons.

Our experiments have shown that by mixing an artificial triglyceride emulsion (Lipomul) with αLP, a Lipomul-αLP complex forms, which floats at d 1.003. This complex, when mixed with αP-I^{131}, showed the same type of interaction as exhibited by chylomicrons. Further, when the labeled Lipomul-αLP-I^{131} complex was injected into dogs, the rate of disappearance of the radioactivity from plasma was similar to that obtained by injecting chylomicrons, labeled in the protein moiety through the interaction chylomicron-αP-I^{131}. In both cases, the rapid process of removal of the triglycerides from circulation was accompanied by the appearance of labeled αLP in plasma, indicating a transfer of the labeled protein moiety from Lipomul or chylomicrons to plasma αLP. Whether this transfer of a labeled protein involves also lipids, cannot be established by these experiments. This possibility is, however, suggested by the experiments of McCandless and Zilversmit (21) and Fredrickson *et al.* (17), which have shown that phospholipids and cholesterol remain in circulation long after chylomicron triglycerides have disappeared from plasma. The similarity in behavior between Lipomul-αLP-I^{131} and labeled chylomicrons, shown by our experiments, may offer indirect evidence that the portion of chylomicrons, which is labeled by interaction with αP-I^{131}, is αLP.

Our results have shown that Lipomul, given intravenously to dogs, combines with a small amount of circulating αLP. Chylomicrons, as we already mentioned, also contain αLP. The question arises as to whether αLP may have any importance in the metabolism of triglycerides. We know, for instance, that αLP, when mixed with an artificial fat emulsion, renders it suitable for hydrolysis by lipoprotein lipase (22, 23), a property which is not exhibited by other serum proteins (4). It seems also established that hydrolysis of triglycerides accompanies their removal from the blood stream (24). It is possible, therefore, that lipoprotein lipase and, consequently, αLP actively participate in the removal of triglycerides from circulation.

From the radioisotope data it appears that αLP and αP are removed from circulation according to a monomial exponential curve suggesting homogeneity of the αLP protein. On the other hand, the starch gel electrophoresis patterns of αLP showed three boundaries each stainable for protein and lipid. Since the electrophoretic mobility of a lipoprotein may vary according to the amount of lipid bound to the protein, it might be assumed that in αLP there are three subfractions containing the same protein but different amount of lipids. However, this hypothesis is ruled out by the fact that three boundaries were also present with αP. Here absence of lipids enhanced significantly the electrophoretic mobility of αP. The presence of three boundaries in αLP and αP may be related to dissociation *in vitro* or *in vivo* of a larger molecule into smaller units and exhibited by starch gel. Another possibility is that the three boundaries are due to protein with different net charge and amino acid composition indicating non-

homogeneity of αLP. Biological homogeneity of αLP may have resulted only because the method we employed was not sensitive enough to detect small differences among similar, but not identical, proteins. This problem is at present under investigation.

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Absence of lipemia clearing factor lipase in human adipose tissue*

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SUMMARY

Clearing factor lipase activity was present in abundance in chicken adipose tissue extracts. No such activity was demonstrable in seven of nine samples of human adipose tissue removed at surgery. Slight lipolytic activity was found in the other two instances.

Lipoprotein lipase, or lipemia clearing factor lipase (which is more accurate terminology [1]), is a heparin-activated enzyme which has been found in rat, beef, and pig heart (2). The adipose tissues of rats, rabbits, and especially chickens contain greater quantities of the enzyme (3). This latter observation led to the suggestion that the major site of lipoprotein lipase activity was in the adipose tissue, where it might function in chylomicron dissolution, and also in the lipolysis of depot fat with resultant free fatty acid release. Clearing factor lipase has also been demonstrated in rat plasma after fat feeding (4), and in the plasma of some humans (5, 6) without the prior injection of heparin, indicating a possible intravascular lipolytic role. The evidence supporting this point of view has recently been summarized (7). In view of these facts, the presence of clearing factor lipase activity in human adipose tissue was investigated.

METHODS

Chicken adipose tissue was obtained as frozen pooled fat from commercial sources. Human material was removed at surgery and immediately frozen. When larger amounts of the human fat were available, an acetone powder extract was prepared within 1 to 2 days. Smaller pieces of tissue were kept frozen for as long as several weeks until a sufficient amount had accumulated. The yield of acetone powder was approximately 200 mg/100 g adipose tissue. A total of 1.5 to 2 kg of human adipose tissue was obtained and extracted. Ammonia extracts of the tissue acetone powders were prepared as described by Korn and Quigley (3) in a concentration of 50 mg/ml. The mixtures and substrates in the different experiments varied as described in the tables. All tubes (Tables 1 and 2) contained 1.4 ml 0.25 M NH_3–NH_4Cl pH 8.5, 0.25 ml 1 M $CaCl_2$, 0.5 ml heparin 0.1 μg/ml. In the experiments using human tissue (Table 2), 2 ml of ammonia extract and 0.5 ml of activated coconut oil were present in all incubation vessels. "Activated" (preincubated with human serum) coconut oil (Ediol®, Schenley) was prepared as outlined by Korn (8). In this study, free fatty acid release was measured in most experiments rather than glycerol, as the former is a more sensitive indicator of partial lipolysis of triglyceride. Free fatty acids were determined by the method of Grossman *et al.* (9) using 1 ml aliquots of the incubation mixtures removed at times indicated in the tables.

RESULTS

The data obtained in experiments with chicken adipose tissue enzyme are shown in Table 1. The values are expressed in microequivalents per milliliter. It is evident that splitting of a simple triglyceride occurred, but lipolysis was markedly enhanced when a lipoprotein substrate, "activated" coconut oil, was used. This activity was partially inhibited by protamine and 0.9 M NaCl (column 7), which inhibit the post-heparin enzyme but have no effect upon pancreatic lipase. The extract was quite active, 100 mg of acetone powder extract resulting in free fatty acid release at a rate of 13 μeq/ml per hour.

* Supported by Grant H-2164 C4 from the National Heart Institute, National Institutes of Health, United States Public Health Service.

TABLE 1. FREE FATTY ACID RELEASE* USING EXTRACT OF CHICKEN ADIPOSE TISSUE. INCUBATION AT 37°

Incubation Time	2.0 ml APE,† 0.2 ml 5% Coconut Oil	2.0 ml 0.025 N NH_4OH, 0.2 ml 5% Coconut Oil	2.0 ml APE, 0.5 ml Act. Coconut Oil‡	Idem + 0.1 ml Protamine	1.0 ml APE, 0.5 ml Act. Coconut Oil, 3 ml 0.15 M NaCl	Idem + 3 ml 2 M NaCl Instead of 0.15 M NaCl
minutes				*10 mg/ml*		
0	1.9	1.9	2.5	2.8	1.7	1.5
15	3.0	1.9	4.8	3.6	2.1	1.8
30	4.2	2.0	8.6	4.3	3.1	1.9
60	7.2	2.0	15.5	4.5	6.1	2.2

* μeq/ml.
† APE = Ammonia extract of tissue acetone powder.
‡ Activated coconut oil.

Table 2 presents the results of studies using nine different batches of human adipose tissue extract, and the same experimental design that gave maximal lipolysis with chicken fat enzyme. No activity is demonstrable in seven experiments. There is very slight release of free fatty acids in two instances (columns 4 and 5). The maximal rate of lipolysis with 100 mg human acetone powder extract was 0.8 μeq per ml per hour. Unfortunately, an insufficient amount of this sample was available and inhibitor studies could not be done. Experiments were also performed using human adipose tissue extracts in which the pH of the buffer and the heparin content were varied, but no increase in hydrolysis occurred. Phosphate buffer (pH 7.6) and physiological saline extracts of acetone powders did not show lipolytic activity when ammonia extracts had previously been inactive. The use of human low density lipoproteins,[1] rich in triglyceride, as fat substrate did not result in any enhancement of lipolytic activity.

DISCUSSION

While this study was in progress, Angervall (10) reported similar experiments in which three of four samples of human adipose tissue extract did not induce lipolysis of an "activated" cream substrate. In one instance he found lipolytic activity, but at a markedly lower rate than that present in chicken fat extracts. Our results with seven individual samples and two pooled batches of human adipose tissue, and using "activated" coconut oil and human low density lipoprotein substrate, are in essential agreement with those of Angervall. Clearing factor lipase activity is not demonstrable in human adipose tissue using an extraction technique which shows abundant enzymatic activity in chicken fat and rat heart. The trace activity we found in a few instances in human extracts has not been identified by inhibitor studies. Furthermore, it may well have come from the capillaries of the adipose tissue rather than from adipose cells themselves. Robinson (11) has presented good evidence that the tissue factor of clearing factor lipase in rabbits is present in capillary walls.

Angervall (10) suggested that the absence of the enzyme in human adipose tissue might be due to the presence of inhibitors in the activating plasma. This explanation would not appear to be valid. In the present study aliquots of the same "activated" coconut oil substrate were used in both human and chicken experiments and excellent activity was present in the latter. With three samples of human extracts no lipolysis of low density lipoproteins occurred, and in these experiments serum was not present. These lipoproteins are an excellent substrate for human clearing factor lipase found in plasma, either endogenously or after the injection of heparin. Finally, Angervall (10) stated that only 22% inhibition of chicken adipose enzyme activity was found when plasma was added.

Using a histochemical technique, Gomori (12) found evidence of lipolytic activity in human adipose tissue. Renold and Marble (13) reported lipolytic activity in extracts of human abdominal and subcutaneous fat using an unphysiologic water soluble substrate, Tween 20. However, just as much of this activity was present in rat liver and in rat pancreas. These observations suggest that the substrate they used was also split by esterases, and therefore the nature of the enzyme involved is uncertain.

A legitimate question arises since our source material

[1] I am indebted to Mr. D. Spector, Institute of Medical Physics, Belmont, California, for supplying the lipoproteins.

TABLE 2. FREE FATTY ACID RELEASE* USING ACETONE POWDER EXTRACT (APE) OF HUMAN ADIPOSE TISSUE. INCUBATION AT 37°

Incubation Time	1 Omental Fat P†	2 Breast Fat I‡	3 Subcut. Abdominal Fat I	4 Subcut. Abdominal Fat I	5 Subcut. Abdominal Fat I	6 Omental Fat P	7 Subcut. Thigh Fat I	8 Subcut. Abdominal Fat I	9 Subcut. Abdominal Fat I
minutes									
0	1.7	1.8	2.4	1.2	2.2	1.7	1.7	1.4	1.5
15	1.6	1.8	lost	1.4	lost	1.8	1.6	1.4	1.3
30	1.7	1.7	2.4	1.6	2.6	1.8	1.8	1.5	1.3
60	1.8	1.9	2.5	1.6	2.6	1.6	1.8	1.5	1.4

* μeq/ml
† P = Pooled from several subjects.
‡ I = Fat from oneindividual.

was obtained from patients who had not eaten for approximately 12 hours. In rats an overnight fast resulted in a 35:1 reduction in the lipase activity of adipose tissue as compared to that of fed rats who also were given sucrose (14). Other investigators (15) have not reported so marked a change due to fasting. They found a 75% reduction in adipose tissue enzymatic activity in rats fasted for 48 hours. The quantitative difference in results may well be the result of the added sucrose. It is unlikely that the absence of clearing factor lipase activity in most of our tissue samples was due to the 12 hours' prior abstinence from food. All the patients except one individual received an intravenous infusion of 5% glucose in physiological saline throughout the operative period. This was started prior to the induction of anesthesia, usually one-half to two hours before the sample of adipose tissue was removed. Furthermore, patients in the post-absorptive state release adequate quantities of clearing factor after the injection of small doses of heparin. Similarly, the endogenous plasma enzyme has been found in some fasting subjects (16). However, in view of our findings, and those of Angervall (10), it is probable that adipose tissue makes little or no contribution to the plasma content of clearing factor lipase in man.

It may not be amiss to draw attention once again to the marked species difference that our data show. The results of experiments with rat or chicken tissues or organs are not automatically transferable to man. It does not appear likely from the studies thus far that adipose tissue clearing factor lipase plays an important role in man. These human adipose tissue findings, however, are not evidence against the probable major role of this enzyme, derived from other tissues, in lipemia clearing.

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The uptake of amino acids by lipids of *pseudomonas aeruginosa*[*†]

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SUMMARY

The lipids of intact *Pseudomonas aeruginosa* resting cells were extracted and the weights of the lipid fractions determined. The phospholipids were found to comprise a major portion (80%) of the ether-soluble lipids. Chromatograms of the phospholipid complex on silicic acid-impregnated paper revealed at least six fractions. Two-dimensional chromatograms of the aqueous acid hydrolyzate of the phospholipids indicated the presence of at least 13 ninhydrin positive compounds. There was a correlation between the uptake of DL-alanine-1-C^{14}, DL-leucine-1-C^{14}, and DL-phenylalanine-3-C^{14} by phospholipids of the resting cells and utilization of these amino acids by the cell as indicated by manometric studies.

Many investigators have described the unique catabolic pathway of carbohydrate metabolism of various species of the genus *Pseudomonas* (1 to 4). It is also well known that the production of observable amounts of acid in a glucose medium varies and is dependent, to a large extent, on the nitrogenous composition of the medium (5 to 8). An investigation was instigated to study the utilization of protein hydrolyzates and amino acids by *P. aeruginosa* in an attempt to develop better procedures for the identification of this gram-negative bacillus in the diagnostic laboratory. During the course of the investigation it was noted that there was excellent correlation between the utilization of amino acids by *P. aeruginosa* and the uptake of the amino acids by the lipids, and in particular the phospholipids of the cell. The results of a study of the lipids of these cells are reported.

METHODS AND MATERIALS

The strain of *P. aeruginosa* used throughout the study was nonpigmented, cytochrome oxidase-positive (9), and produced observable amounts of acid in a glucose broth medium containing 1% peptone. Resting cells were obtained by growing the culture in a nutrient broth medium containing yeast extract (2 g/150 ml broth) at 37° for 18 hours on a rotary shaking machine which described a circle of one inch diameter at 250 rpm. The cells were harvested by centrifugation and washed several times with cold 0.1 M pH 7.0 phosphate buffer. The cell suspensions were standardized by optical density, viable cells counts, as well as wet and dry weight determinations. Oxygen consumption of the resting cells was measured by conventional Warburg techniques described by Umbreit *et al.* (10).

Uptake of C^{14}-labeled Amino Acids. Resting cells were suspended in pH 7.0 phosphate buffer (0.1 g wet weight/ml) containing 0.5 mg/ml of the amino acid to be studied. The cells were incubated for varying periods of time at 37° on the rotary shaker. Following incubation, the cell suspension was placed in an ice bath and centrifuged at 4°. The cells were washed three or four times with large volumes of cold buffer until the radioactivity of the wash buffer was negligible.

Lipid Extraction. The cells were resuspended in an Erlenmeyer flask containing 200 ml of a chloroform-methanol 2/1 (v/v) solvent mixture and shaken at room temperature for 18 hours. This technique, as originally described by Folch *et al.* (11), was intended for use with macerated or homogenized cells. Following extraction, the cellular residue was separated from the solvent mixture by filtration and the chloroform-methanol extract evaporated to dryness *in vacuo*. The lipids were taken up in petroleum ether and washed with distilled water. The phospholipids were precipitated by the addition of acetone. The acetone-in-

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soluble fraction was reprecipitated several times from petroleum ether and washed thoroughly with distilled water or saline, or both.

The phospholipids were hydrolyzed in aqueous 6 N HCl for 24 hours at 100°. The aqueous hydrolyzate, following repeated evaporation, was chromatographed on Whatman #1 filter paper for identification of the nitrogenous compounds. Duplicate chromatograms were run on all samples. One chromatogram was used for the detection of compounds while corresponding areas were cut from the duplicate but untreated chromatogram for the detection of radioactivity. The radioactivity, determined with the use of a windowless flow counter, was corrected for self-absorption of the paper.

All chromatograms were developed by an ascending technique (12). The solvent systems used to develop the chromatograms were: *n*-butanol-acetic acid-water (13), phenol-water (14), methanol-benzene-*n*-butanol-water (15), and chloroform-methanol plus 2% water or diisobutyl ketone-acetic acid-water (16) or both for silicic acid-impregnated paper.

RESULTS

The results listed in Table 1 show the average weights of the lipid fractions obtained from *P. aeruginosa* cells. It is of interest to note that the phospholipids comprise approximately 80% of the ether-soluble lipids. The lipid content of these cells could be increased by growing the culture in a nutrient medium containing specific phospholipids (phospholipids extracted from *P. aeruginosa* cells). It is obvious from the results shown in Table 2 that while the amounts of the lipid fractions could be increased, the per cent composition of the fractions remained constant.

TABLE 1. RESULTS OF WEIGHT STUDIES OF *P. Aeruginosa*

Fraction	Weights
Wet cells	5.55 g (4 to 7 × 10^{11} viable cells/gm)
Dry cells	1.0079 g (18% ± wet weight)
Ether-soluble lipids	0.0945 g (1.7% ± 0.1% wet weight)
Phospholipids (acetone-insoluble)	0.0707 g (80% ± 5% ether-soluble lipids)
Acetone-soluble lipids	0.0238 g (20% ± 5% ether-soluble lipids)

It has previously been found in this laboratory (17) and elsewhere (18) that there is a variation in the utilization of different amino acids by resting cells of *P. aeruginosa*. The ability of 18 different amino acids studied to stimulate O_2 consumption of these cells ranged from 18.5 μl O_2 uptake/90 min/mg dry weight for DL-methionine to 74.1 μl for DL-serine. The results listed in Table 3 compare the stimulation of O_2 consumption by *P. aeruginosa* of three representative amino acids. To compare the uptake of these amino acids by the lipids of *P. aeruginosa* cells, 0.5 mg/ml of DL-leucine-1-C^{14}, DL-phenylalanine-3-C^{14}, and DL-alanine-1-C^{14} were added to pH 7.0 phosphate buffer containing a suspension of 0.1 g wet weight resting cells per ml. The cells were incubated on the rotary shaker at 37° for 90 minutes. Following incubation, the cells were washed thoroughly with cold buffer and the lipids

TABLE 2. COMPARATIVE WEIGHTS OF LIPID FRACTIONS EXTRACTED FROM *P. Aeruginosa* CELLS GROWN IN CONTROL MEDIUM AND PHOSPHOLIPID-ENRICHED MEDIUM

Lipid Fraction	*P. Aeruginosa* Cells Grown in Control Medium*		*P. Aeruginosa* Cells Grown in Phospholipid-Enriched Medium†	
	Weight	Ether-Soluble Fraction	Weight	Ether-Soluble Fraction
	mg	*per cent*	*mg*	*per cent*
Ether-soluble	66.7	100	102.1	100
Acetone-insoluble phospholipid	56.4	84.6	83.1	81.4
Acetone-soluble	8.0	12	9.5	3.9

* 4.19 g wet weight cells from 450 ml broth.

† 5.47 g wet weight cells from 600 ml broth containing 0.215 mg of phospholipid/ml.

TABLE 3. EFFECT OF THREE REPRESENTATIVE AMINO ACIDS ON THE O_2 UPTAKE OF *P. Aeruginosa*

Amino Acid*	O_2 Uptake†	
	Endogenous	Plus Substrate
DL-Leucine	21.8	32.5
DL-Phenylalanine	20.0	40.7
DL-Alanine	21.2	70.3

* Amino acid concentration: 0.5 mg/ml incubation medium, incubated in Warburg at 37° in phosphate buffer pH 7.0.

† μl/10 minutes/mg dry weight.

extracted as described. The uptake of the C^{14}-labeled compounds by the lipid fractions is shown in Table 4. The figures shown represent μg of amino acids taken up based on their specific radioactivity. These results point out the correlation between the utilization of amino acids by *P. aeruginosa* and the uptake of amino acids by the phospholipid complex of the cell. The phospholipids of heat-killed cells did not take up radioactivity, nor did the phospholipids extracted from *P. aeruginosa* and shaken *in vitro* with C^{14}-labeled amino acids take up radioactivity.

Phospholipids extracted from resting cells which had been incubated with DL-alanine-1-C^{14} and DL-leucine-1-C^{14} were chromatographed by the technique described by Westley *et al.* (15). Figure 1 shows the results of chromatograms of the phospholipids extracted from *P. aeruginosa* cells following incubation with DL-leucine-1-C^{14}. Similar results were obtained with phospholipids from DL-alanine-1-C^{14} incubated resting cells. In every instance the radioactivity traveled with the phospholipids (rhodamine G positive). C^{14}-labeled amino acids added to the extracted phospholipids separated on chromatography. The aqueous acid hydrolyzates of the phospholipids chromatographed by this technique or with *n*-butanol-acetic acid-water indicated that the radioactivity was concentrated in the area corresponding to the R_f value of the respective amino acid with which the cells had been incubated. Figure 2 shows the results of radioactive phospholipids developed on silicic acid-impregnated paper (16). The chromatograms were run in triplicate to determine the R_f values of the ninhydrin, choline, and rhodamine G-positive compounds as well as the radioactive areas. It should be noted that the R_f values shown will vary slightly with different lots of silicic acid-impregnated paper. It was of interest to find that with either solvent system used (chloroform-methanol plus 2% water or diisobutyl ketone-acetic acid-water) all of the radioactivity remained with the rhodamine G-ninhydrin-positive spots on or near the origin (R_f <0.05).

TABLE 4. UPTAKE OF C^{14}-LABELED AMINO ACIDS BY THE PHOSPHOLIPIDS OF *P. Aeruginosa*

Fraction	DL-Amino Acid	Fraction Weight	Amino Acid Taken Up*	Amino Acid in 100 mg of Fraction
		mg	*μg*	*μg*
Petroleum ether-soluble lipids	Leucine-1-C^{14}	47.5	17.8	37.5
	Phenylalanine-3-C^{14}	47.9	23.4	48.9
	Alanine-1-C^{14}	72.0	32.1	44.6
Acetone insoluble lipids†	Leucine-1-C^{14}	35.2	5.32	15.1
	Phenylalanine-3-C^{14}	34.1	7.00	20.0
	Alanine-1-C^{14}	58.6	21.3	36.4

Cells incubated 90 minutes at 37° on shaker in phosphate buffer pH 7.0; amino acid concentration: 0.5 mg/ml; cell concentration: 0.1 g wet weight/ml; incubation medium: total volume 30.0 ml.

* μg amino acid taken up based on total amount of radioactivity found in the fraction: 1 μg DL-alanine-1-C^{14} = 5940 cpm; 1 μg DL-leucine-1-C^{14} = 1070 cpm; 1 μg DL-phenylalanine-3-C^{14} = 966.5 cpm.

† Phospholipids.

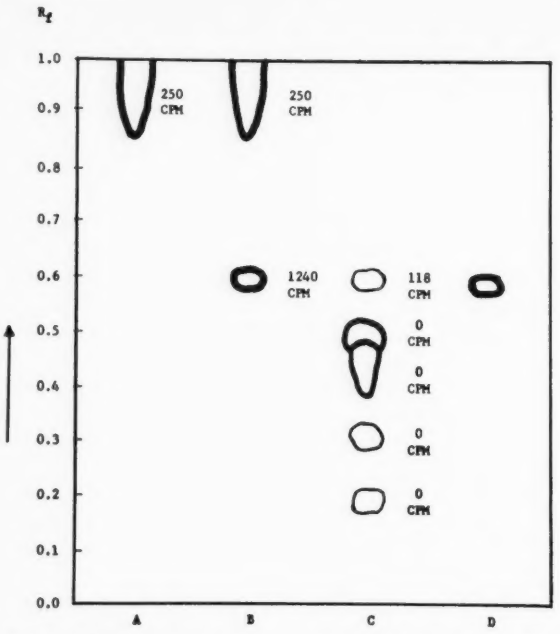

FIG. 1. Chromatographic demonstration of the binding of C^{14}-labeled amino acid to phospholipids extracted from resting cells of *P. aeruginosa*. Solvent system: Methanol-benzene-*n*-butanol-H_2O (2/1/1/1, v/v). (A) Phospholipids extracted from cells incubated with DL-leucine-1-C^{14}. (B) Phospholipids extracted from cells incubated with DL-leucine-1-C^{14} plus DL-leucine-1-C^{14} added to the partially purified phospholipids. (C) Aqueous acid hydrolyzate of phospholipids extracted from cells incubated with DL-leucine-1-C^{14}. (D) DL-leucine control. Phospholipid spots above R_f 0.8 both ninhydrin- and rhodamine G-positive, spots below R_f 0.8 ninhydrin-positive. CPM = counts per minute.

Two-dimensional chromatography of the water-soluble acid hydrolyzate of phospholipids extracted from control resting *P. aeruginosa* cells revealed (Fig. 3) that there were at least 13 ninhydrin-positive compounds present. Most of these ninhydrin-positive spots have been identified by the use of known compounds.

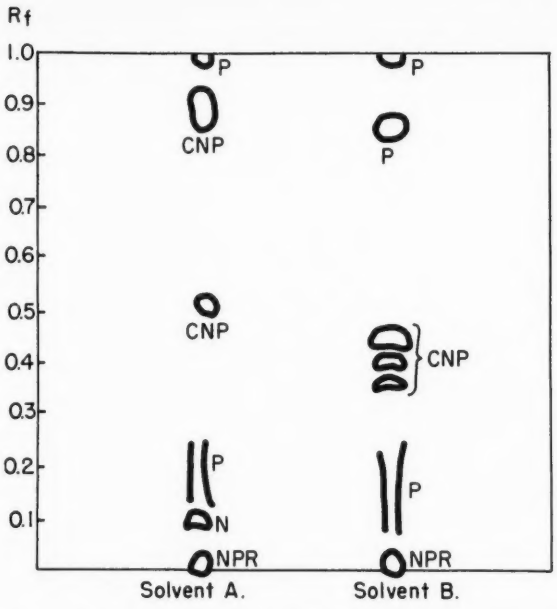

FIG. 2. Chromatographic separation on silicic acid-impregnated paper of phospholipids extracted from *P. aeruginosa* cells incubated with DL-leucine-1-C^{14}. Solvent A: Chloroform-methanol (3/1, v/v) + 2% H_2O. Solvent B: Diisobutyl ketone-acetic acid-H_2O (40/30/7, v/v). Identification of spots. P = rhodamine G-positive; N = ninhydrin-positive; C = choline-positive; R = radioactive area.

DISCUSSION

Current investigations involving lipids extracted from various tissues and microorganisms reveal that there is yet a great deal to be learned about the phospholipids and their role in metabolism. Haining *et al.* (19) working with mitochondria of rat tissues have shown that the degree of amino acid uptake by the lipid fraction of mitochondria varied with the type of compounds studied, but that they were taken up without degradation or alteration. Hendler (20) concluded from his studies with hen oviduct that the lipids appeared to be an intermediate carrier of amino acids entering the cell. He characterized the lipid amino acid "bond" as being highly labile in contrast to the stable bond linking an amino acid component of a nucleic acid fraction. Hokin and Hokin (21) have proposed, as a result of their studies, a mechanism of transport of zymogens across lipid membranes of the cells, with the phospholipids acting as some type of intermediate in this mechanism. Hunter and Goodsall (22) concluded from their studies with *Bacillus megaterium* that phospholipid-amino acid complexes may be involved in the transfer of amino acids from the site of amino acid activation to the site of protein synthesis. They found that following incorporation studies and extraction of phospholipids from cells of *B. megaterium*, the radioactivity incorporated did not separate from the phospholipids when chromatographed on silicic acid-impregnated paper.

The data presented in the present study support the existence of a phospholipid-amino acid complex as a part of the lipid component of *P. aeruginosa*. Repeated experiments confirmed the observations that resting cells of *P. aeruginosa* varied in their ability to utilize different amino acids, and this variation was reflected in the uptake of C^{14}-labeled amino acids by the phospholipid complex of the cell. Furthermore, it would appear that amino acids are taken up only by phospholipid complex of metabolically active cells. The phospholipids of heat-killed cells or partially purified phospholipids extracted from the cells do not take up C^{14}-labeled amino acids.

Two-dimensional chromatograms run on the aqueous acid hydrolyzates of phospholipid complexes extracted from *P. aeruginosa* cells indicated the presence of at least 12 ninhydrin-positive compounds in addition to choline. Chromatograms of aqueous acid hydrolyzates of the phospholipid complexes extracted from resting

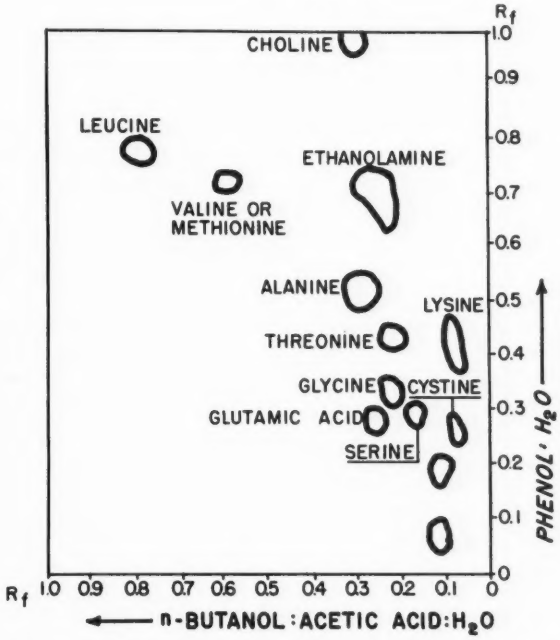

FIG. 3. Two-dimensional chromatogram of the aqueous acid hydrolyzate of phospholipids extracted from normal resting cells of *P. aeruginosa*. All spots with the exception of choline were ninhydrin-positive.

cells previously incubated with an amino acid showed an increase in the intensity of the ninhydrin-positive spot which corresponded to the R_f value of the amino acid with which the cells were incubated.

The identification of the phospholipids in this study was based on their solubility properties in various lipid solvents. The authors are aware of the possibility that these microorganisms might have a large content of novel lipids not related to the phospholipids.

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Propionic acid as a precursor in the biosynthesis of animal fatty acids*

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SUMMARY

The carboxyl carbon of propionate was found to be a poor precursor for the synthesis of fatty acids in the rat. The data indicate that propionate as a three-carbon unit is not incorporated into long-chain fatty acids by the intact rat to any appreciable extent. The results also suggest that the conversion of propionate to long-chain fatty acids in surviving adipose tissue occurs primarily by a mechanism involving the decarboxylation of the propionate. These data are consistent with the concept that the major pathway of lipogenesis in adipose tissue is not different from pathways described for the liver. A small fraction of the propionate is converted to long-chain fatty acids in adipose tissue *in vitro* by a mechanism that involves the use of propionate as an intact three-carbon unit, but in any case, its significance in the intact animal would appear to be negligible.

The liver was long considered as the major, if not the sole, site of lipogenesis in the mammalian organism (1). However, recently Favarger and Gerlach (2) and Favarger (3) have presented evidence that indicates that the liver does not play a major role in this process. These authors found that hepatic lipogenesis can account for only 4% of the total lipogenic activity of the mouse and that most of the remaining fatty acid synthetic activity is carried out in the adipose tissue.

In the last few years important advances in understanding the biochemical mechanism by which fatty acids are synthesized in the animal organism have been made (4, 5); however, most of these studies have been carried out on liver enzyme systems. It is of interest, therefore, that Feller and Feist (6, 7, 8) have presented considerable evidence indicating that adipose tissue can convert propionic acid as an intact three carbon unit into long-chain fatty acids, and have proposed a pathway of lipogenesis in adipose issue that is different from those described for liver (9). They also have shown that this pathway does not occur in the liver, because in this organ the carboxyl carbon of propionate is not incorporated into long-chain fatty acids. If all of the above assumptions are true and if the pathway proposed by Feller and Feist is a major one, it would be anticipated that most of the fatty acid synthesis in the intact animal would be found to occur via a pathway other than currently accepted ones. Since this pathway involves the use of propionic acid as a three-carbon unit, it would seem to be a corollary that administration of propionic acid labeled in the carboxyl position should lead to a large incorporation of the label into the body fatty acid.

METHODS

In Vivo Experiments. Male rats of the Wistar strain (inbred at Tufts for about 20 years) were kept at 25° for 10 days before the experiment and were allowed to eat the standard diet of the following composition: 25% casein, 10% lard, 50.95% glucose, 3% salt mix (10), 5% cellulose, 6% brewer's yeast, 0.04% cod liver oil concentrate, and 0.01% alpha tocopherol. The rats used in the experiments weighed 230 ± 10 g and two groups were fasted for 6 hours prior to the experiment. One group was given by stomach tube 1 g of triacetin plus 1.9 g dextrose containing a tracer dose of sodium acetate-1-C^{14}.[1] Another group was given 1 g of tripropionin plus 1.9 g dextrose containing sodium propionate-1-C^{14} in a tracer dose. The rats were then placed in a metabolism cage and their res-

* This work was accomplished under Air Force Contract AF41(657)321 monitored by the Alaskan Air Command, Arctic Aeromedical Laboratory, and was also aided by Research Grant No. RG4989 from the National Institutes of Health and by a grant from the Whitehall Foundation.

[1] The C^{14}-labeled compounds used in these experiments were purchased from the New England Nuclear Corporation, Boston, Mass.

piratory CO_2 was collected for 24 hours by the methods described in a previous paper (1). During the course of this metabolism study the rats had access to the diet described above.

The collected respiratory $C^{14}O_2$ was analyzed for its C^{14} content by a liquid counting method (11). At the end of the experiment the rats were killed by a blow on the head and the carcass was analyzed for lipids (12). The C^{14} content of the fatty acids was determined by pipetting 15 mg of the C^{14} fatty acids dissolved in $CHCl_3$ into a cupped nickel planchet and drying until the last trace of $CHCl_3$ had disappeared. The fatty acids were then counted at this mass and the counts were corrected to infinite thinness by a conversion factor. The C^{14} content of the labeled acetic acid and propionic acid was also measured (11).

A third group of rats was treated in a somewhat different manner. These rats were fed until the experiment was started. They were then injected intraperitoneally with 1 ml of saline containing either sodium acetate-1-C^{14} or sodium propionate-1-C^{14}. They were at once placed in wire cages and either fed or fasted. The respiratory $C^{14}O_2$ was not collected. The rats were sacrificed after 24 hours and the lipids isolated and analyzed from C^{14} content as described above.

In Vitro Experiments. Male rats of the above strain and male white Swiss mice (obtained from Mrs. Woodman, of Wayland, Mass.) were kept at 25° for 10 days on our standard diet. They were sacrificed by decapitation and the epididymal fat pad was excised and placed in a petri dish of Krebs-Henseleit bicarbonate buffer solution (13). A weighed piece of this adipose tissue (chosen randomly) was then placed into an incubation flask (14). The adipose tissue was kept at room temperature throughout this preincubation manipulation.

TABLE 1. METABOLISM OF GASTROINTESTINALLY-ADMINISTERED ACETATE-1-C^{14} AND PROPIONATE-1-C^{14} BY RATS

No. Rats Used*	Labeled Substrate†	Per Cent Labeled Substrate Converted to	
		CO_2	Fatty Acids
5	Acetate-1-C^{14}	78.8 ± 2.47‡	5.9 ± 0.75
5	Propionate-1-C^{14}	79.5 ± 7.07	0.3 ± 0.08

* Rats weighed 230 ± 10 g.

† 1 g of triacetin, 1.9 g of glucose and tracer dose of sodium acetate-1-C^{14} or 1 g of tripropionin, 1.9 g glucose and tracer dose of sodium propionate-1-C^{14} were administered by stomach tube; the rats were killed 24 hours later.

‡ S. E. (Note that in the case of the CO_2 analysis, the average value refers to only 4 rats.)

The main compartment of the incubation flask contained the adipose tissue, 4.5 ml of Krebs-Henseleit bicarbonate buffer containing glucose, 100 mg/100 ml, and 0.5 ml of saline containing 5 μmoles of the labeled substrate. The system was gassed for about 30 seconds with 95% O_2–5% CO_2 gas mixture, sealed and incubated for 3 hours at 37.5° with continuous shaking.

The $C^{14}O_2$ formed during the incubation was recovered by the method of Chernick *et al.* (15) and its C^{14} content was then determined (11).

At the end of the incubation the adipose tissue was removed from the incubation flask and saponified in 30% KOH made up in a solvent of 50% ethanol. The fatty acids were then extracted from this hydrolyzate of adipose tissue by a method described earlier (16). The C^{14} content of the fatty acid was determined by a previously described method (17).

The C^{14} content of the labeled acetic acid and propionic acid was then determined (11).

RESULTS

The results of experiments in which rats were given by stomach tube either (*a*) sodium acetate-1-C^{14}, triacetin, glucose, or (*b*) sodium propionate-1-C^{14}, tripropionin, glucose, are recorded in Table 1. More than 75% of the C^{14} content of each of the labeled compounds appeared in the respiratory CO_2 during the 24 hours of the experiment. Significant quantities of C^{14} from the acetate-1-C^{14} appeared in the long-chain fatty acids of the body while C^{14} from propionate 1-C^{14} was almost completely absent. These results were surprising in light of the *in vitro* mouse experiments of Feller and Feist (6 to 9), and it seemed possible that these findings were due to the trapping by the liver of the labeled propionate entering it from the portal vein, thus preventing the propionate from reaching the adipose tissue. To circumvent this possibility to some extent, carboxyl-labeled propionate was administered intraperitoneally (Table 2), and the rats were then either fed or fasted for 24 hours. Very little of the C^{14} of the administered propionate-1-C^{14} appeared in the body's fatty acids. In a recently published investigation (18) Feller reported that about 2% of the C^{14} of intraperitoneally administered carboxyl-labeled propionate was found in the body fatty acids of mice fasted for 24 hours following the isotope administration.

Since the results of our *in vivo* studies are not consistent with the *in vitro* results of Feller and Feist (6 to 9), it was decided that the *in vitro* system should be reinvestigated. As shown in Table 3, rat liver is able to convert large amounts of acetate-1-C^{14} into fatty

TABLE 2. METABOLISM OF INTRAPERITONEALLY-ADMINISTERED PROPIONATE-1-C^{14} BY RATS

Nutritional State of the Rat Following Propionate-1-C^{14} Administration*	Per Cent Propionate-1-C^{14} Converted to Fatty Acids
Fed	0.5
Fed	0.3
Fasted	0.2
Fasted	0.1

* Rats weighed 230 ± 10 g; propionate-1-C^{14} was administered intraperitoneally in a tracer dose and the rats were killed 24 hours later.

acids but is unable to convert any of the carboxyl of propionate into long-chain fatty acids. On the other hand, while adipose tissue uses considerable amounts of the carboxyl carbon of acetate for lipogenesis, it uses very little of the carboxyl carbon of propionate for the purpose.

This finding led to an investigation of the conversion of propionate-1-C^{14}, propionate-2-C^{14}, and propionate-3-C^{14} to long-chain fatty acids by surviving adipose tissue (Table 4). Propionate-2-C^{14} and propionate-3-C^{14} entered fatty acids to a considerable extent, and at practically the same rate. In contrast, the conversion of propionate-1-C^{14} was very small. Since these results are not in agreement with those of Feller and Feist (7) in their studies with mice, propionate-1-C^{14} and propionate-2-C^{14} metabolism experiments were carried out on mouse adipose tissue (Table 5). It can be seen that in the mouse, as in the rat, propionate-2-C^{14} appears to be a much better substrate than propionate-1-C^{14} for lipogenesis.

The incubation medium used by Feller and Feist differs from ours in two particulars, namely, it contains more glucose than our medium and it contains 0.01 M succinate while our medium contains none. Therefore experiments using the succinate-containing medium were carried out (Tables 6 and 7). Although the results are not quantitatively identical to those found in our medium, it can be seen that propionate-2-C^{14} is still a better precursor of long-chain fatty acids than is propionate-1-C^{14}. It should be noted that the succinate depressed propionate metabolism in the case of rat adipose tissue, but that it promoted mouse adipose tissue lipogenesis, while still not abolishing the difference in the behavior of the 1 and 2 carbons.

TABLE 3. ACETATE-1-C^{14} AND PROPIONATE-1-C^{14} METABOLISM BY SURVIVING RAT LIVER SLICES AND ADIPOSE TISSUE

No. Rats Used*	Labeled Substrate†	Tissue Used‡	Per Cent C^{14} Converted to	
			CO_2	Fatty Acids
5	Acetate-1-C^{14}	Liver	33.7 ± 2.61§	4.8 ± 1.51
	Propionate-1-C^{14}	Liver	66.2 ± 1.46	0.0 ± 0.0
	Acetate-1-C^{14}	Adipose tissue	5.8 ± 0.32	4.0 ± 1.12
	Propionate-1-C^{14}	Adipose tissue	14.8 ± 0.77	0.6 ± 0.09

* Each substrate was incubated with liver and adipose tissue from each of the five rats; therefore results are a comparison of incubation flasks, not a comparison of different rats.

† The incubation system contained the tissue in 5 ml of Krebs-HCO_3^- buffer containing 100 mg/100 ml glucose and 5 μmoles of the labeled substrate; system was incubated at 37.5° for 3 hours.

‡ 250 mg of tissue used.

§ S. E.

TABLE 4. PROPIONATE METABOLISM BY SURVIVING RAT ADIPOSE TISSUE

No. Rats Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	19.9 ± 3.06§	0.9 ± 0.25
	Propionate-2-C^{14}	7.0 ± 0.94	7.5 ± 1.50
	Propionate-3-C^{14}	7.0 ± 0.88	7.6 ± 1.50

* Each substrate was incubated with adipose tissue from each of the five animals; therefore results are a comparison of incubation flasks, not a comparison of different animals.

† See footnote, Table 3.

‡ 250 mg of adipose tissue were used in each flask.

§ S. E.

DISCUSSION

The experiments presented above indicate that the

TABLE 5. PROPIONATE METABOLISM BY SURVIVING MOUSE ADIPOSE TISSUE

No. Mice Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	17.6 ± 1.46§	1.7 ± 0.32
	Propionate-2-C^{14}	6.7 ± 0.58	7.3 ± 0.61

* See footnote, Table 4.

† See footnote, Table 3.

‡ 200 mg of adipose tissue used in each flask.

§ S. E.

TABLE 6. PROPIONATE METABOLISM BY SURVIVING RAT ADIPOSE TISSUE INCUBATED IN A SUCCINATE-CONTAINING MEDIUM

No. Rats Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	7.2 ± 0.30§	0.6 ± 0.06
	Propionate-2-C^{14}	3.3 ± 0.30	2.3 ± 0.35

* See footnote, Table 4.

† The incubation system contained the tissue in 5 ml of the incubation medium of Feller and Feist (7) containing 5 μmoles of the labeled substrate. The system was incubated at 37.5° for 3 hours.

‡ 250 mg of tissue were used.

§ S. E. (Note that the value of 7.2 refers to only 4 rats.)

carboxyl carbon of propionate is not used to an appreciable extent in the biosynthesis of long-chain fatty acids in the intact rat. As a corollary of this finding, it can be concluded that in the intact organism a very small fraction of fatty acid synthesis occurs via a pathway utilizing propionate as an intact molecule. This is true whether the propionic acid is ingested or is administered to the animal by intraperitoneal injection.

In the case of surviving adipose tissue, it is evident that the carboxyl carbon of acetate is far more readily incorporated into long-chain fatty acids than is the carboxyl label of propionate. Furthermore, on the basis of varying the position in which propionate is labeled, it is evident that propionate-2 or -3 carbons are far better precursors of long-chain fatty acids than is the carboxyl carbon. It seems evident, therefore, that even in adipose tissue studied *in vitro*, most of fatty acid synthesis is not occurring via a pathway that involves the conversion of the propionate molecule as a unit into long-chain fatty acids, but rather by pathways not inconsistent with those described for the liver by Brady (4) and Wakil *et al.* (5).

TABLE 7. PROPIONATE METABOLISM BY SURVIVING MOUSE ADIPOSE TISSUE INCUBATED IN A SUCCINATE-CONTAINING MEDIUM

No. Mice Used*	Labeled Substrate†	Per Cent‡ C^{14} Converted to	
		CO_2	Fatty Acids
5	Propionate-1-C^{14}	15.7 ± 1.26§	3.1 ± 0.60
	Propionate-2-C^{14}	4.4 ± 0.10§	15.5 ± 1.85

* See footnote, Table 4.

† See footnote, Table 6.

‡ 100 or 150 mg of tissue used; all values are corrected to that of 100 mg of tissue.

§ S. E. (Note that the value of 4.4 refers to only 4 mice.)

It will be clear that some of the data presented in this paper lead to conclusions which are at variance with those advanced by Feller and Feist (7, 8, 18). In the case of the *in vivo* experiments, the possibility existed that the variance could be attributed to species difference since Feller (18) used mice and we used rats. However, in the case of the *in vitro* experiments, similar results were obtained by us with mice as with rats. It is true that minor differences between our technique and those of Feller and Feist exist, such as the composition of the diet, the region of the body from which the fat was taken, and the method of slicing and chilling the tissue. These differences, however, were not of a nature, it seems to us, to explain the discrepancies in our results.

Our data with propionate-1-C^{14} indicate that in isolated adipose tissue a small fraction of the propionate enters long-chain fatty acids by a mechanism involving the use of propionate as an intact three-carbon unit. This finding is compatible with the possibility that the pathway suggested by Feller and Feist (9) is operative to a limited extent in adipose tissue. However, it seems probable from our data that such a pathway has little or no activity in the intact organism.

Since the submission of this manuscript for publication, Favarger and Gerlach (19) have published a report of their studies on the synthesis of fatty acids from propionate by the intact mouse. They find that three times as much C^{14} is incorporated into fatty acids when propionate-2-C^{14} is administered intravenously to mice than when propionate-1-C^{14} is used. Propionate-1-C^{14} is primarily incorporated in the odd-chain fatty acids and on basis of degradation studies the authors have concluded that intact propionate molecules are incorporated in the ω position of odd-chain acids and that the 2 and 3 carbons of propionate are incorporated via acetyl-CoA throughout the carbon skeleton of odd- and even-chain fatty acids. Our results are completely compatible with the findings and conclusions of Favarger and Gerlach.

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Fat synthesis in cell-free preparations of the locust fat-body

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SUMMARY

It was shown that cell-free preparations of the fat-body of the migratory locust, *Locusta migratoria*, incorporated acetate into fatty acids in the presence of ATP, CoA, glutathione, Mg^{++}, TPN, malonate, α-ketoglutarate, and $KHCO_3$. The major fatty acid component synthesized was palmitic acid. The newly synthesized acids were esterified by the system with glycerol as glycerides and phospholipids. Mitochondria were not required for synthesis. Fat-body homogenates could also activate and decarboxylate malonate and form malonic acid by CO_2 fixation.

Fat metabolism in higher animals has been extensively studied for many years, and several of the intermediate steps of fat synthesis and degradation have been elucidated (1). Relatively little is known about fat metabolism in insects. Most insects have a fat-body which combines some of the functions of liver and adipose tissue of higher animals (2). As experimental specimen, the migratory locust, *Locusta migratoria*, was chosen. Weis-Fogh (3) had previously shown that during flight locusts utilized fat stored in the fat-body. From differences between the RQ values found in resting insects and during sustained flight, he calculated that 85% of the total energy spent was supplied by the oxidation of lipids. Recently it was shown by Meyer *et al.* (4) that a particulate fraction from the flight muscles of the desert locust could completely oxidize fatty acids *in vitro* under suitable conditions. Since locusts renew their fat reserves during rest and feeding, it was to be expected that the fat-body would synthesize lipids from suitable precursors. Clements (5) showed, indeed, that intact sheets of previsceral fat-body converted, *in vitro*, acetate, glucose, and amino-acids into fat. To study the mechanism of these reactions, cell-free extracts of fat-body tissue were prepared, and the incorporation of acetate into fatty acids was investigated. A preliminary account of this work has been presented before the Israel Chemical Society (6).

METHODS

Preparation of Cell-free Systems. Locusts (*Locusta migratoria*) were bred in glass cages and fed grass and oatmeal. The cages were kept at 28° and 70% humidity. Fat-body tissue was removed from male and female locusts 8 to 15 days after the last molt. The tissue obtained from a single insect was homogenized with 0.6 ml of a buffer containing 0.085 M K_2HPO_4, 0.009 M KH_2PO_4, 0.010 M $KHCO_3$, and 0.002 M GSH.[1] The homogenate was centrifuged at 400 × *g* for 5 minutes and then filtered through a small pad of cotton to remove fat which accumulated on top. To obtain subcellular particles, the homogenate was further centrifuged at 20,000 × *g* for 20 minutes. The supernatant was decanted, filtered to remove fat, and used without further manipulations. The precipitate was suspended in fresh buffer by homogenization. Usually a concentrated suspension of particles was prepared.

Enzymatic Assays. The method for assay of fatty acid synthesis was carried out as described by Wakil *et al.* (7). Acetate-1-C^{14} was incubated with cofactors and enzyme for 2 hours at 30° under air. The reaction was stopped by addition of 10% ethanolic KOH and the mixture was saponified for 3 hours. Following acidification, the long-chain fatty acids were extracted into petroleum ether (b.p. 40°–60°). Aliquots were taken from the extract for plating and counting. In some experiments when esterified lipids were isolated, the reaction was stopped by addition of 10 volumes ethanol-ether (3/1, v/v) followed by heating to the boiling point of the mixture.

To measure the decarboxylation of malonate-1-C^{14} and malonate-2-C^{14}, incubations were carried out in

[1] Abbreviations: GSH = glutathione; ATP = adenosine triphosphate; CoA = coenzyme A; DPN and TPN = diphospho- and triphosphopyridine nucleotides, respectively.

Thunberg tubes. The reaction was stopped by addition of 0.2 ml 5 N H_2SO_4 from the side bulb. After 10 minutes the tubes were evacuated. To trap liberated $C^{14}O_2$, the air was passed through a 10% NaOH solution. Na_2CO_3 was converted to $BaCO_3$ for plating and counting.

$C^{14}O_2$ fixation was measured as described by Tietz and Ochoa (8). To identify the labeled acids formed, the reaction was stopped by addition of H_2SO_4 to a final concentration of 3N. The protein-free supernatant was mixed with Celite® (9) and the acids extracted with ether in a Soxhlet apparatus for 3 hours.

To measure acetate and malonate activation, the substrates were incubated with cofactors, hydroxylamine, and enzyme. Formation of acethydroxamic and malonomonohydroxamic acid was estimated by forming the colored iron complex (10). To isolate the hydroxamates formed, the reaction was stopped by addition of 10 volumes of ethanol. The protein-free supernatant was evaporated under reduced pressure and the residue extracted into a small volume of ethanol.

Separation of Lipids. Phospholipids were separated from a mixture of lipids by precipitation with acetone (11) after addition of carrier yolk-phospholipids. To isolate cholesterol, the acetone supernatant was extracted with petroleum ether after saponification and cholesterol precipitated from the extract with digitonine (12) after addition of carrier cholesterol. Neutral-fat fatty acids were extracted from the saponified mixture after acidification.

After it had been shown that only negligible amounts of acetate were incorporated into unsaponifiable matter, the acetone supernatant (after precipitation of phospholipids) was passed through a MgO-Celite® column (11) and glycerides and free fatty acids were eluted with acetone and methanol, respectively. Free fatty acids were obtained from the purified phospholipids and glycerides after saponification.

Paper Chromatographic Procedures. Long-chain fatty acids were resolved according to Buchanan (13) with acetic acid-88% formic acid-30% H_2O_2 (6/1/1, v/v) as developing solvent. Chromatograms were run for 18 hours at 30°.

Acetic and malonic acids were separated according to Isherwood and Hanes (14), with propanol-30% ammonia (6/4, v/v) as developing solvent.

Acethydroxamic and malonomonohydroxamic acids were separated according to Fink and Fink (15) with butanol-acetic acid or water-saturated phenol, and identified by spraying with ethanolic $FeCl_3$ (16).

C^{14} Assays. Lipid material was plated on planchets which were lined with lens paper. Not more than 1 mg material per cm^2 was plated to avoid self-absorption corrections. $BaCO_3$ and cholesterol digitonide were pipetted directly onto the planchets and dried under an infrared lamp. Self-absorption corrections were made when necessary. To locate C^{14}-labeled material on a chromatogram, the paper strip was cut into 1 cm pieces and each piece was counted separately. An end window GM-counter was used throughout.

MATERIALS

The following substances were obtained from commercial sources: crystalline ATP, DPN, and TPN (Sigma Chemical Company); CoA (Pabst Laboratories). Acetate-1-C^{14}, $BaC^{14}O_3$, ethylmalonate-1-C^{14}, and ethylmalonate-2-C^{14} were obtained from the Radiochemical Centre, Amersham, Bucks, England. Ethylmalonate was saponified before use. Since the malonate-1-C^{14} sample contained 2% contamination and the malonate-2-C^{14} 50% contamination, the acids were purified by chromatography on Whatman No. 3MM. paper (14).

RESULTS

Conditions for Fatty Acid Synthesis by Fat-Body Homogenates and Extracts. To determine optimal conditions for fatty acid synthesis from acetate by fat-body homogenates, the latter were incubated with acetate-1-C^{14} in the presence of different substrates and cofactors, and the recovery of C^{14} in fatty acids was estimated. The results are summarized in Table 1.

TABLE 1. COMPONENTS REQUIRED FOR FATTY ACID SYNTHESIS

Cofactor Omitted	Acetate Incorporated into Fatty Acid
	μmoles
None*	0.33
ATP	0.05
CoA	0.14
GSH	0.17
DPN	0.33
TPN	0.19
$MgCl_2$	0.01
$MnSO_4$	0.32
Malonate	0.01
α-ketoglutarate	0.20
$KHCO_3$	0.08

* The complete system contained (in μmoles): acetate 5 (80,000 cpm acetate-1-C^{14}), ATP 5, CoA 0.1 mg, GSH 5, DPN 0.5, TPN 0.5, $MgCl_2$ 10, $MnSO_4$ 0.5, malonate 20, α-ketoglutarate 10, $KHCO_3$ 10, and 0.6 ml (2 mg protein) homogenate in a total volume of 1 ml. The pH was adjusted to 7.0 by addition of HCl.

ATP, $MgCl_2$, $KHCO_3$, and malonate were required for synthesis; CoA, GSH, and TPN further stimulated the system; DPN and $MnSO_4$ had no effect. Malonate could not be replaced by any intermediate of the glycolytic or Krebs cycle. However, addition of some of these intermediates in the presence of malonate caused further stimulation. The best results were obtained with α-ketoglutarate. Citrate and isocitrate had no effect; glucose-6-phosphate showed some stimulation (Table 2).

TABLE 2. REPLACEMENT OF MALONATE BY OTHER SUBSTRATES

Additions	Acetate Incorporated into Fatty Acids
	μmoles
None*	0.01
α-ketoglutarate	0.09
Citrate	0.06
Malonate	0.25
Malonate + α-ketoglutarate	0.40
Malonate + citrate	0.25
Malonate + G6P	0.33
Malonate + α-glycerophosphate	0.27

* The complete system contained acetate and cofactors as mentioned in the footnote to Table 1. The following substances were added when indicated: α-ketoglutarate, citrate, glucose-6-phosphate, and α-glycerophosphate, 10 μmoles each; malonate 20 μmoles, and 0.6 ml. homogenate (3 mg protein) in a total volume of 1 ml.

The optimal concentrations of the essential components of the system were as follows: ATP 2.5 to 10 μmoles, CoA 0.1 mg or more, GSH 5 μmoles or more, TPN 0.5 μmole, $MgCl_2$ 5 to 10 μmoles, $KHCO_3$ 10 to 20 μmoles, malonate 15 to 25 μmoles, and α-ketoglutarate 10 μmoles. The optimal pH was 7.0. Whereas at pH 7.0, 0.44 μmole of acetate was incorporated into fatty acids, at pH 6.5 only 0.20 μmole, and at pH 7.8, 0.25 μmole of acetate was used.

Particles were not required for fatty acid synthesis. Under optimal conditions the particle-free supernatant was as active as the original homogenate in incorporating C^{14}-acetate into fatty acids.

Isolation of Lipid Components Synthesized by Fat-Body Homogenates and Extracts. To determine the lipid components which were synthesized by the fat-body preparations, phospholipids, glycerides, free fatty acids, and unsaponifiable matter were isolated as described under Methods. As can be seen from Table 3, only negligible amounts of C^{14} were recovered in

TABLE 3. C^{14} CONTENT OF ISOLATED LIPID COMPONENTS

Lipid Fraction	C^{14} Recovered in Fraction	
	Homogenate	Supernatant
	cpm × 10^3	*cpm* × 10^3
Lipid mixture (total)	127	121
Phospholipids	23.3	11.6
Glycerides	102	108.5
Unsaponifiable matter	.625	
Cholesterol digitonide	.045	
Free fatty acids	.950	

Incubation mixture as described in the footnote to Table 1. 800,000 cpm acetate-1-C^{14} were added per tube. Two tubes were pooled before the component lipids were separated.

unsaponifiable matter and the cholesterol digitonide precipitated from it; C^{14}-acetate was incorporated only into fatty acids. The newly synthesized fatty acids were esterified with glycerol and did not accumulate as free acids. The fat-body homogenate esterified 18% as phospholipids and 81% as glycerides; the mitochondria-free supernatant, 10% as phospholipids and 90% as glycerides.

To determine the composition of the newly synthesized fatty acids which were contained in the phospholipid and glyceride fractions, the acids were isolated and an aliquot was separated by paper chromatography. With the highly labeled glyceride-fatty acids a clear separation of the acids was obtained (Fig. 1).

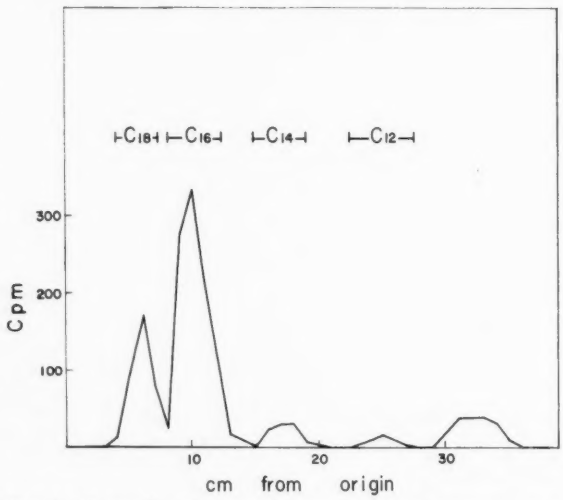

FIG. 1. Separation by paper chromatography of glyceride-fatty acids synthesized by locust homogenate.

The fatty acids of the phospholipid fraction did not contain enough C^{14} to obtain a significant distribution of the counts when a small amount of material was used, as required by the paper chromatographic method. As can be seen from Table 4, C^{14} was found in all *n*

TABLE 4. Composition of Glyceride-Fatty Acids

Fatty Acid	C^{14} Recovered in Fractions as Per Cent of Total	
	Homogenate	Supernatant
Stearic (octadecanoic)	22	2.5
Palmitic (hexadecanoic)	60	55
Myristic (tetradecanoic)	5	18
Lauric (dodecanoic)	2	3.5
Shorter and unsaturated acids	11	21.0

long-chain fatty acids with an even number of carbon atoms from 12 to 18. The major fatty acid component was palmitic acid. A considerable amount of radioactivity traveled with the fastest peak representing fatty acids which contain less than 12 carbon atoms and unsaturated acids. Except for minor differences, the pattern of labeling in the glyceride-fatty acids obtained from homogenate and particle-free supernatant was similar.

Activation of Acetate and Malonate. The presence of activating enzymes for acetate and for malonate in locust fat-body preparations could be readily shown when these substrates were incubated in the presence of cofactors, hydroxylamine, and enzyme. In the particle-free supernatant, hydroxamic acid formation occurred in the absence of added substrates. This activation was retained even when the supernatant was first dialyzed overnight. Addition of acetate stimulated hydroxamic acid formation only slightly. Malonate was not activated by this system (Table 5). The formation of hydroxamic acid was dependent on the addition of ATP and CoA. The hydroxamic acids formed by the supernatant were separated by chromatography on paper. In the absence of added substrates two spots were identified: one corresponded to acethydroxamic acid, the second moved faster and corresponded to hydroxamic acids of longer chain length. In the presence of acetate the acethydroxamic acid spot was augmented. When the particles were tested for activating enzymes, addition of malonate, but not of acetate, resulted in the formation of hydroxamic acid.

TABLE 5. Formation of Hydroxamic Acids

Additions*	Hydroxamic Acid Formed	
	Supernatant	Particles
	μmoles†	*μmoles*†
None	0.87	0.02
Acetate	1.06	0.02
Malonate	0.85	0.68

* The reaction mixture contained (in μmoles): hydroxylamine low in salt content, adjusted to pH 8.0 500, ATP 10, $MgCl_2$ 10, CoA 0.1 mg, GSH 5 and 0.5 ml of enzyme (2.5 mg protein) in a total volume of 1 ml; 20 μmoles of acetate or malonate were added as indicated. Incubated 1 hour at 30°.

† Results expressed as μmoles of acethydroxamic acid.

The hydroxamic acid formed was separated by chromatography and identified as malonomonohydroxamic acid.

Carbon Dioxide Fixation. To study the effect of CO_2 on fatty acid synthesis, $C^{14}O_2$ fixation by fat-body homogenates was investigated. $C^{14}O_2$ was readily fixed. Addition of acetate stimulated the fixation only very slightly; propionate was slightly inhibitory (Table 6).

TABLE 6. Fixation of $C^{14}O_2$ by Fat-Body Homogenates

Additions*	CO_2 Fixed
	μmoles
None	0.34
Acetate	0.42
Propionate	0.26

* The reaction mixture contained (in μmoles): ATP 5, $MgCl_2$ 10, CoA 0.1 mg, GSH 5 and 0.6 ml homogenate (pH 7.8) in a total volume of 0.8 ml. Ten μmoles of acetate or propionate were added as indicated. Fourteen μmoles of $K_2C^{14}O_3$ containing 200,000 cpm in 0.2 ml were tipped in from the side bulb after 5 minutes' equilibration, and the incubation continued for 2 hours.

More $C^{14}O_2$ was fixed at pH 7.8 (0.31 μmole) than at pH 7.0 (0.22 μmole). Both mitochondria and supernatant were required for $C^{14}O_2$ fixation; each fraction alone was inactive. The $C^{14}O_2$ which had been fixed was not incorporated into fatty acids; it was found in ether-extractable acids. These were separated by chromatography. Of the counts, 91% were recovered in malonic acid, 9% in acetic acid.

Metabolism of Malonate. To study the effect of malonate on fatty acid synthesis, the metabolism of malonate by fat-body preparations was investigated. When malonate-1-C^{14} was added instead of acetate-1-

C^{14} to the reaction mixture, C^{14} was recovered in $BaCO_3$ and fatty acids. To test for a possible connection between the decarboxylation of malonate and fatty acid synthesis, the incorporation of acetate-1-C^{14} and of malonate-1-C^{14} into CO_2 and fatty acids by homogenates, supernatant, and particles was compared. As can be seen from Table 7, acetate was not oxidized under the experimental conditions, but it was readily incorporated into fatty acids by the homogenate and particle-free supernatant. Malonate-1-C^{14} was decarboxylated by the homogenate and particles; only trace activity was found in the supernatant. The homogenate also utilized malonate-1-C^{14} for fatty acid synthesis, but to a smaller extent than acetate. Although the incorporation of acetate-1-C^{14} by the supernatant was higher than by the homogenate, utilization of malonate by the supernatant was negligible. The particles converted some malonate into fatty acids, and the synthesis of fatty acids from malonate exceeded that from acetate. However, in all experiments more C^{14} was found in $BaCO_3$ than in fatty acids; decarboxylation seems to exceed the utilization of malonate for fatty acid synthesis. Addition of acetate to a reaction mixture which contained malonate-1-C^{14} did not affect the metabolism of malonate by fat-body preparations. These experiments were repeated with malonate-2-C^{14}. As expected, no C^{14} was recovered in $BaCO_3$. The pattern of C^{14} incorporation into fatty acids was similar to that obtained with malonate-1-C^{14}. However, the recovery of C^{14} was lower than expected and did not amount to twice the activity found with malonate-1-C^{14}.

TABLE 7. METABOLISM OF ACETATE-1-C^{14} AND OF MALONATE-1-C^{14}

Preparation	Additions*	C^{14} Incorporated into	
		$BaCO_3$	Fatty Acids
		μmoles	*μmoles*
Homogenate	Acetate-1-C^{14} + malonate	0.0	0.21
	Malonate-1-C^{14}	0.10	0.05
	Malonate-1-C^{14} + acetate	0.09	0.04
Supernatant	(1-C^{14}) acetate + malonate	0.0	0.31
	(1-C^{14}) malonate	0.01	0.02
	(1-C^{14}) malonate + acetate	0.01	0.01
Particles	(1-C^{14}) acetate	0.0	0.02
	(1-C^{14}) malonate	0.15	0.04
	(1-C^{14}) malonate + acetate	0.17	0.05

* Conditions of incubation as described in the footnote to Table 1; 210,000 cpm malonate-1-C^{14} added as indicated.

Decarboxylation of malonate by the particles was further studied. In the absence of ATP, decarboxylation dropped from 0.19 μmole to 0.07 μmole. A requirement for CoA could also be shown. The decarboxylation was faster at pH 7.8 than at pH 7.0; at pH 7.0 it dropped to 0.09 μmole.

DISCUSSION

It has been shown that the biosynthesis of fatty acids in cell-free preparations of locust fat-body required the addition of ATP, CoA, GSH, Mg^{++}, TPN, $KHCO_3$, malonate, and α-ketoglutarate. These requirements are very similar to those reported for liver (17) and mammary gland (18) extracts. A similar system was also obtained by Zebe and McShan (19) from the fat-body of the moth *Prodenia eridania*. The similarity in substrate and cofactor requirement of the different systems suggests that a similar mechanism for fatty acid synthesis is operative. CoA, ATP, and Mg^{++} are therefore required for acetyl-CoA formation. The requirements of malonate and α-ketoglutarate for fatty acid synthesis were described in mammary gland extracts (18), and it was suggested that α-ketoglutarate reduced added pyridine nucleotides. The effect of malonate could not be explained. The requirement of $KHCO_3$ for fatty acid synthesis in liver extracts was first described by Gibson *et al.* (20). In this system $KHCO_3$ acted catalytically. It was required for the formation of malonyl-CoA from acetyl-CoA (21). In the presence of TPNH, malonyl-CoA condensed with acetyl-CoA to yield fatty acyl-CoA (22); the newly fixed CO_2 was lost in the condensation reaction. To study the effects of malonate and $KHCO_3$ in the locust system, the C^{14}-labeled substrates were used. The presence of activating enzymes for acetate and malonate, a system which decarboxylated malonate and a system which fixed CO_2 and formed malonate, were found in the locust fat-body homogenate. There was, however, no correlation between the distribution of the fatty acid synthesizing system and the above-mentioned enzymes. Whereas acetate activation and fatty acid synthesis occurred in the particle-free supernatant, particles were required for the activation and decarboxylation of malonate. A particulate system from kidney, which activated and decarboxylated malonate, was previously described by Nakada *et al.* (23). CO_2 was fixed only in the presence of both particles and supernatant. Since activation of acetate occurred in

the supernatant, it seems likely that the mitochondria catalyze the fixation of CO_2 to acetyl-CoA. The finding that malonate and bicarbonate are required in a system which apparently cannot metabolize them is difficult to explain at present. Further work is being done to elucidate these effects.

The finding that fat-body homogenates converted acetate into fatty acids and not into cholesterol was expected, since it is known that intact insects cannot synthesize sterols from small molecules (24) and require their addition in the diet. In analogy to the cell-free system of Zebe and McShan (19), the locust fat-body homogenate synthesized all *n* long-chain fatty acids with an even number of carbon atoms and also some unsaturated acids. Palmitic acid contained about 60% of the total label. In contrast to the above-mentioned system, in the locust fat-body preparations all the newly synthesized fatty acids were esterified with glycerol as glycerides and phospholipids. An analysis of the fatty acid components of *Locusta migratoria*, published by Hilditch (25), showed that oleic, linoleic, and linolenic acid amounted to 65% of the total fatty acids. This distribution differs markedly from the distribution of labeling found in the fatty acids which were synthesized *in vitro* by fat-body extracts. It is of interest to test whether under suitable conditions the extracts can convert saturated fatty acids into unsaturated acids, or whether the locust, like higher animals, requires an external supply of linoleic and linolenic acid.

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The *in vivo* effect of digitoxin on rat heart phosphatides*

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SUMMARY

Digitoxin was shown to increase the specific activity of heart ventricle phosphatides in rats receiving simultaneously radioactive orthophosphate and digitoxin as compared to control rats not receiving digitoxin. The increase in specific activity was demonstrated in both lecithin and phosphatidyl ethanolamine. The other heart muscle lipids were not investigated. Glycerol and ethanol, which are constituents of the vehicle in which the digitoxin is dissolved, do not alter the specific activity of the heart muscle phosphatides. The vehicle itself (without digitoxin) has some activity.

The biochemical mechanism of action of cardiac glycosides on heart muscle has not been sufficiently investigated. The *in vitro* effect of cardiac glycosides on acetylcholine esterase and adenosine triphosphatase has been discussed by Proctor *et al.* (1), who present the concept that cardiac glycosides may act by inhibiting adenosine triphosphatase with the result that more ATP[1] is available for muscular work. The *in vitro* work of Grisolia (2), who described a potentiating effect of digitoxin on dinitrophenol uncoupling of oxidative phosphorylation, is difficult to reconcile with this hypothesis.

In view of the high caloric value of lipids, the abundant concentration of lipids in heart muscle (3, 4), and the lipophilic nature of cardiac glycosides, it was considered possible that these drugs might increase the turnover of certain lipids with the concomitant increase in ATP production. Experiments were thus carried out in which white rats were given simultaneously P^{32}-labeled orthophosphate and digitoxin, and the specific activity of the total phosphatides, lecithin, and phosphatidyl ethanolamine were determined and compared with those in control rats not receiving digitoxin. These experiments show that digitoxin does produce an increase in the specific activity of the phosphatides. This increase in specific activity was found in both lecithin and phosphatidyl ethanolamine.

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[1] ATP signifies adenosine triphosphate.

METHODS

Experiments 1 and 2: Control rats were injected subcutaneously with 200 μc of P^{32}-orthophosphate contained in 0.5 ml of 0.001 M phosphate buffer pH 7.4. Experimental rats were injected with the same amount of radioactive phosphate and were also injected intraperitoneally with 180 μg of digitoxin (Varick) contained in 0.9 ml of vehicle.[2]

Experiment 3: Rats were given 200 μc of orthophosphate as explained in experiment 1. In addition, these rats were injected intraperitoneally with 0.9 ml of 40% glycerol in water. This experiment was designed to test the effect of glycerol in the vehicle.

Experiment 4: Rats were given 200 μc of orthophosphate as explained in experiment 1. In addition, these rats were injected intraperitoneally with 0.9 ml of 40% ethanol in water. This experiment was designed to test the effect of the alcohol which is in the vehicle.

Experiment 5: Control rats were given 200 μc of radioactive phosphate as explained in experiment 1, and also given 0.9 ml of vehicle intraperitoneally. This vehicle contains all the ingredients in the digitoxin preparation except the digitoxin. Experimental rats were given the same amount of radioactive phosphate and 0.9 ml of digitoxin preparation (vehicle plus 180 μg of digitoxin). This experiment was designed to test the effect of the vehicle.

[2] The vehicle in which the digitoxin is dissolved contains the following: Polyethylene glycol 300, 28.6%; glycerol, 43.8%; benzyl alcohol, 4.0%; ethyl alcohol, 5.0%; and water to make 100%.

TABLE 1. THE EFFECT OF DIGITOXIN ON RAT HEART MUSCLE PHOSPHATIDES

	Specific Activity (cpm/μg of P)						
		Total Phosphatides		Lecithin		Phosphatidyl Ethanolamine	
	No. of Animals	Mean	Standard Error*	Mean	Standard Error*	Mean	Standard Error*
Expt. 1							
Control	3	30.7	3.8	37.7	5.0	29.7	5.8
Digitoxin + vehicle	3	51.3	3.8	52.0	5.0	59.7	5.8
Expt. 2							
Control	5	28.6	2.9	33.8	3.8	22.8	4.5
Digitoxin + vehicle	5	47.4	2.9	44.6	3.8	43.0	4.5
Expt. 3							
Glycerol	5	30.6	2.9	36.2	3.8	27.0	4.5
Expt. 4							
Ethanol	3	30.0	3.8	41.3	5.0	29.3	5.8
Expt. 5							
Control + vehicle	4	39.2	3.2	44.0	4.3	34.5	5.0
Digitoxin + vehicle	5	49.0	2.9	53.0†	5.0	‡	
Expt. 1 + 2							
Controls	8	29.4	2.3	35.2	3.0	25.4	3.5
Expt. 1 + 2 + 5							
Digitoxin + vehicle	13	48.9	1.8	49.2§	2.5	49.2§	3.5

* Standard errors based upon pooled estimates of the standard deviation for each lipid sample.
† Value based upon 4 animals.
‡ Data not included because samples were lost because of an accident.
§ Lecithin mean based upon 12 animals; phosphatidyl ethanolamine mean based on 8 animals.

Male rats (200 to 225 g) were used in all experiments. The rats were sacrificed 24 hours after injection. This time interval was chosen because it represents a plateau region of the specific activity time curve (5). Total lipid extracts were made of the heart ventricles. The lipids were extracted with methanol-ether and then washed three times with isotonic saline. The total lipids were dissolved in isoamyl alcohol-benzene 1/1 (v/v) at a concentration of 40 mg/ml. Appropriate aliquots were taken for the determination of the specific activity of the total phosphatides and for quantitative paper chromatography. The latter method was used to determine the specific activity of lecithin and phosphatidyl ethanolamine. The quantitative paper chromatography has been described previously (6). The data of these experiments are summarized in Table 1.

RESULTS

The data in Table 1 demonstrate that in three different experiments the digitoxin preparation markedly increases the specific activity of the total rat heart ventricle phosphatides. Furthermore, the data show that this increase occurs in both lecithin and phosphatidyl ethanolamine. The effect is greater with phosphatidyl ethanolamine than with lecithin.[3]

Since the vehicle in which the digitoxin is dissolved contains several compounds, it was desirable to test some of these compounds and the vehicle independently. The results of these experiments, which are also given in Table 1, show that glycerol and ethanol had no effect on the turnover of the heart phosphatides, but that the vehicle did produce some stimulation. The total digitoxin preparation, however (vehicle plus digitoxin), produced the greatest effect. When the differences among the means are compared with their standard errors, it can be seen that digitoxin does increase the rate of incorporation of radioactive phosphorus in the rat heart phosphatides.

[3] It was not ascertained whether the plasmalogen or diester fraction of these phosphatides was stimulated. Our analysis (unpublished data) shows that 8% of the lecithin and 23% of the phosphatidyl ethanolamine in rat heart is of the plasmalogen form.

DISCUSSION

These experiments support the hypothesis that, in part, the action *in vivo* of digitoxin on heart muscle is to increase the turnover[4] of certain phosphatides. This increased turnover may be coupled with ATP production and hence yield energy for muscle work. It remains to be determined whether digitoxin affects the other lipids of heart muscle. It also remains to be determined at what time interval the effect of digitoxin is most pronounced.

Digitoxin has its therapeutic action on the failing heart and gives little or no physiological response on the normal heart.[5] The experiments reported in this paper were done on normal rats. The difficulty in producing artificial heart failure in rats did not allow the same study on such rats.

The reason why digitoxin is more effective on the failing heart than on the normal heart may be related to the rate of ATP production. If the rate of ATP production in the normal heart is just sufficient or in excess of its demands, then any further increase in its production would have no effect on the animal. On the other hand, if the ATP production is less than the required amount for a normal functioning heart (as might prevail in the failing heart), then an increase in its production would have a marked effect on the animal. It may be possible that the increased incorporation of isotope into the phosphatides is associated with an increased rate of oxidation of these lipids. This increased oxidation might yield more ATP by the usual metabolic pathway for fatty acid oxidation in the mitochondria.

The effect observed in these experiments may also be considered to involve membrane phosphatides and hence a permeability process might be implicated rather than ATP production. The increased incorporation may be associated with the transport of a specific cation or substrate across the cell membrane.

[4] It is assumed that the increase in specific activity of the phosphatides means an increased turnover rate, and hence both the rate of synthesis and degradation are increased. It is also possible that the digitoxin stimulates the uptake of inorganic phosphate or increases the specific activity of phosphorylcholine and phosphorylethanolamine or their respective cytidine nucleotide derivatives. Furthermore, the turnover of the phosphorylated base and the diglyceride moieties of the glycerol phosphatides may or may not be the same.

[5] The therapeutic dose of digitoxin given to humans is between 1.2 to 1.6 mg/day on the first day, and thereafter 0.1 to 0.2 mg/day. The dose given to the rats in this experiment on a weight basis may appear to be in excess, but it is difficult to extrapolate from the human to the rat.

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Notes on Methodology

A modified preparative ultracentrifuge technique for separating human serum lipoproteins*

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» Preparative ultracentrifugation of human serum to separate lipoproteins into recoverable fractions which are amenable to quantitative analysis of their constituents is a valuable laboratory tool. It is desirable for laboratories using direct methods of measuring cholesterol and phospholipids to be able to quantify without having to rely on time-consuming dialysis or extraction. It is the purpose of this paper to show that sodium sulfate, rather than sodium chloride or a sodium chloride-potassium bromide mixture, should be used to adjust solvent density if a direct procedure for measuring cholesterol is to be used.

The preparative ultracentrifugal procedure was comparable to that of Havel *et al.* (1). The Spinco Model L was used with a 40.3 rotor at 38,000 rpm for 16 hours at 2°. Two 2-ml serum samples for each subject were centrifuged simultaneously, one at a solvent density of 1.006 g/ml, the other at 1.063 g/ml.

Aqueous Na_2SO_4 solutions for controlling solvent density:

Density 20°	Concentration, g/liter	Molarity
1.006	8.6	0.061
1.063	76.73	0.54
1.12	144.85	1.02

The 1.006 g/ml sample was prepared by measuring the serum into a lusteroid tube and then filling with 1.006 g/ml salt solution. The 1.063 g/ml sample was prepared by diluting the serum with an equal volume of 1.12 g/ml salt solution and then filling the tube with the salt solution of 1.063 g/ml. The Spinco Tube Slicer was used to recover the fractions.

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† Established Investigator, American Heart Association.

Chemical analyses were performed on three of the four fractions obtained, namely: $d > 1.063$, $d < 1.063$, and $d < 1.006$. The cholesterol procedure used was that described by Zlatkis *et al.* (2) with the color reagent modification described by Rosenthal *et al.* (3). The phospholipid procedure used was that described by Zilversmit and Davis (4). Reproducibility and recovery of cholesterol and phospholipids from the ultracentrifugally separated lipoproteins using sodium sulfate were comparable to those reported by Havel *et al.* (1).

To determine the effects of added salts on the direct determination of cholesterol, equal portions of standard, of serum, and of the lipoprotein fractions were diluted with an equal volume of either NaCl or Na_2SO_4 solutions. The lipoprotein fractions were separated using Na_2SO_4. The concentrations of the added salt solutions are shown in the abscissas in Figure 1. They are expressed in density units so that comparison with solvent densities used in ultracentrifugation may be made. Sodium chloride caused very significant errors, whereas sodium sulfate had virtually no effect on cholesterol values. Neither salt affected phospholipid determinations.

It is not understood why increasing the sodium chloride affects the cholesterol determination. One hypothesis is that the excess sodium chloride reacts with the ferric chloride to increase the concentration of a compound with the formula Na_2FeCl_5 (5). When separated from aqueous solution, other comparable alkaline metallic chlorides crystallize with $FeCl_3$ to give compounds with the general formula $M_2FeCl_5 \cdot H_2O$. It may be that under the anhydrous conditions of the test, cholesterol takes the place of the water and that basically the compound measured is $Na_2FeCl_5 \cdot$cholesterol. As the chloride concentration increases, the formation of this cholesterol compound would rise to

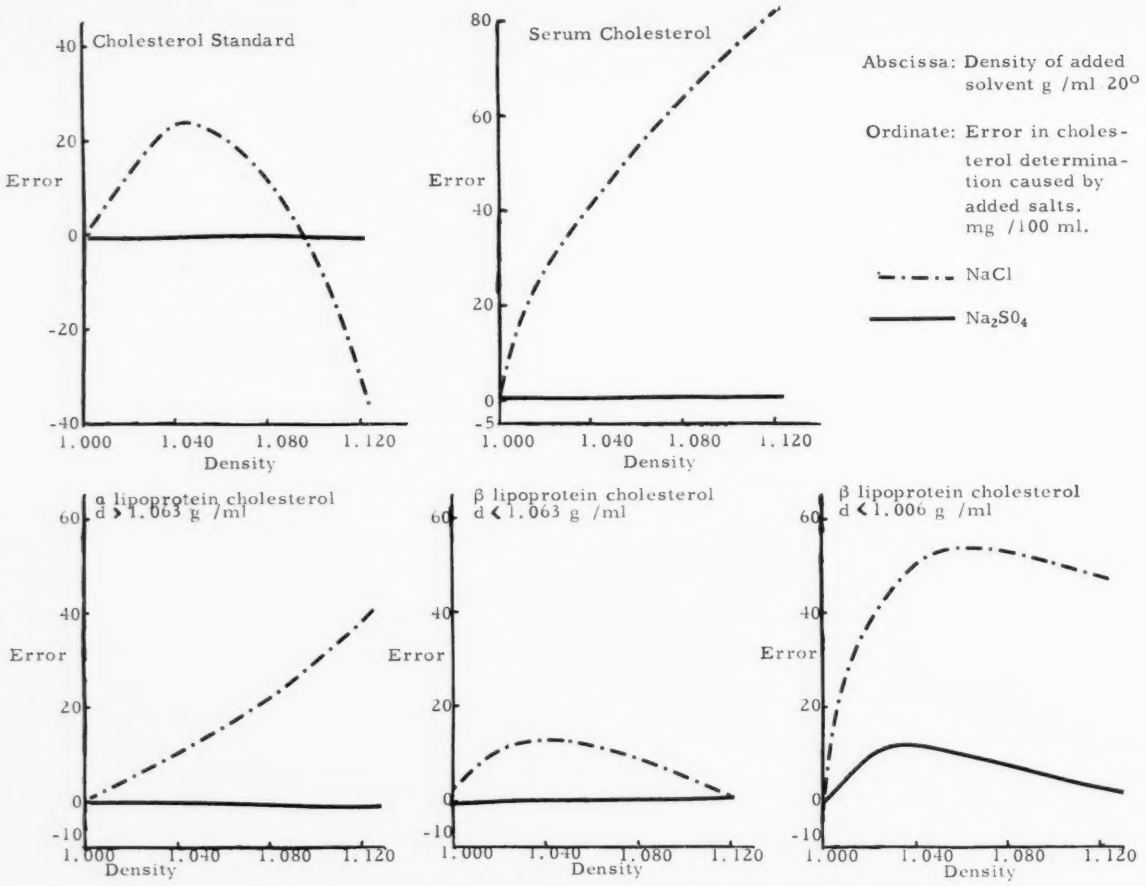

FIG. 1. Error in cholesterol determinations caused by added salts.

a maximum, then decrease as the formation of the complex compound would be inhibited by the excess chloride competing to form Na_3FeCl_6 or some higher complex. In Figure 1 the curve given by the standard solution would be explained by this hypothesis.

The whole serum and the d > 1.063 lipoprotein cholesterol curves do not follow this hypothesis unless the following reasoning is used. They both have a relatively high concentration of protein which must successfully compete for the excess sodium chloride, thus preventing the formation of a maximum color in the chloride range given. The fact that protein concentrations in the serum lipoprotein fractions d < 1.063 and d < 1.006 are relatively small and that maxima are reached, tends to confirm this reasoning.

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Estimations of serum cholesterol from protoporphyrin-stained electrophoretic strips*

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» Lipophylic dye measurements of serum lipoproteins separated by filter paper electrophoresis cannot be converted into chemical units (1). Therefore several investigators (2 to 5) have proposed techniques whereby lipoproteins fractionated electrophoretically are estimated in terms of cholesterol. These methods, however, require preparation of duplicate electrophoretic strips, one of which is stained for lipids. One strip can then be used as a marker for identification of alpha- and beta-lipoprotein areas on the other strip. The staining of reference strips is not only time-consuming but limits by one-half the number of determinations that can be carried out simultaneously in the electrophoretic cell. There is some degree of uncertainty in locating lipoprotein zones in this manner, since adjacent strips rarely demonstrate identical electrophoretic migrations. However, it is not feasible to elute cholesterol directly from strips stained with Oil Red O or Sudan Black B. These dyes, when dissolved in 60% ethanol, are capable of extracting considerable amounts of lipid material from strips during the staining process (1). In addition, the solubility of the lipophylic dyes in the usual cholesterol extractants might be expected to interfere appreciably with subsequent colorimetric measurements. To circumvent these difficulties a fluorescent technique has been employed in our laboratories, whereby electrophoretic strips are stained for lipid with protoporphyrin in a pure aqueous system (6). The use of protoporphyrin as a lipid stain was first described by Kôsaki *et al.* (7, 8, 9) and was utilized by Sulya and Smith (10) to locate purified lipids applied to filter paper. More recently it has been observed that serum cholesterol may be eluted directly from stained strips, since protoporphyrin does not interfere with colorimetric analysis of this lipid.

Filter paper strips are dried in an oven at 110° for 15 minutes following the electrophoretic separation of 50 μl of serum. The strips are then immersed for 5 minutes in 0.05 N HCl containing 5.0 mg/100 ml of commercially available purified protoporphyrin.[1] Rinsing is achieved by placing the filter papers in distilled water for another 5 minutes. For maximum sensitivity Sulya and Smith (10) suggest that protoporphyrin-stained lipid chromatograms be viewed under ultraviolet light while still damp. However, in our hands, exposure of serum electrophoretic strips to 110° for approximately 15 minutes enhances ultraviolet fluorescence. These strips retain fluorescent qualities for several days, while those allowed to dry at room temperature fade appreciably in a matter of hours.

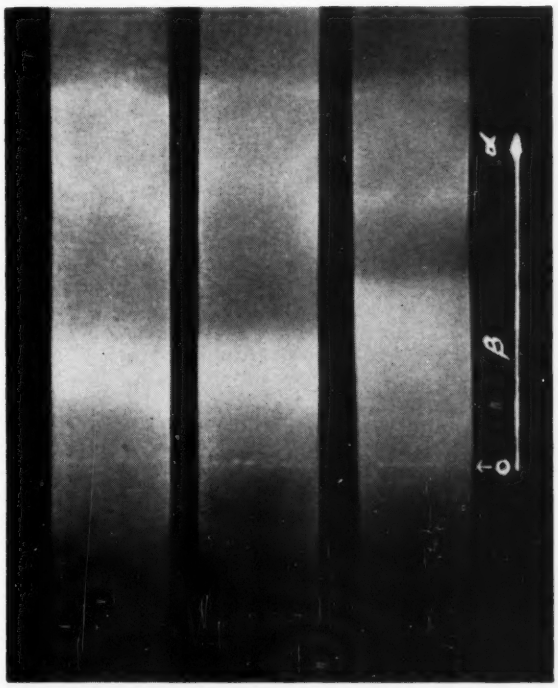

Fig. 1. Fluorescent electrophoretic strips stained with an acid solution of protoporphyrin showing location of α- and β-lipoproteins.

In ultraviolet light the protophorphyrin-treated electrophoretic strips prepared from 50 μl of serum show the major lipid-bearing proteins as brilliant red fluorescent zones against a blue background (Fig. 1). In

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† Medical Student Research Fellowship, National Institutes of Health.

[1] California Corporation for Biochemical Research, Los Angeles, Calif.

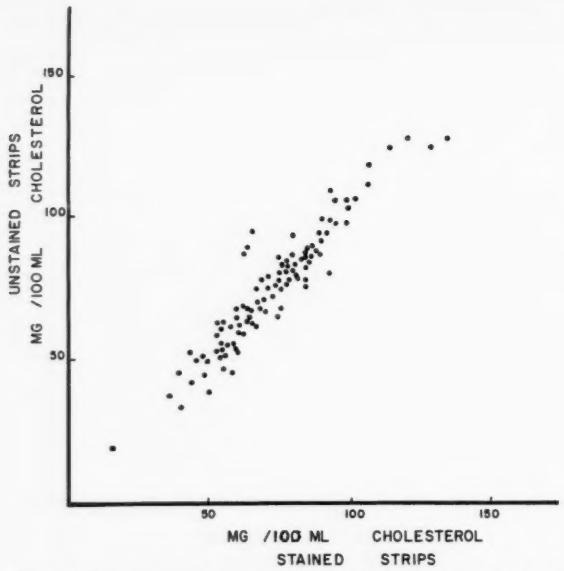

FIG. 2. Comparison of α-lipoprotein cholesterol values obtained from protoporphyrin-stained and unstained electrophoretic strips.

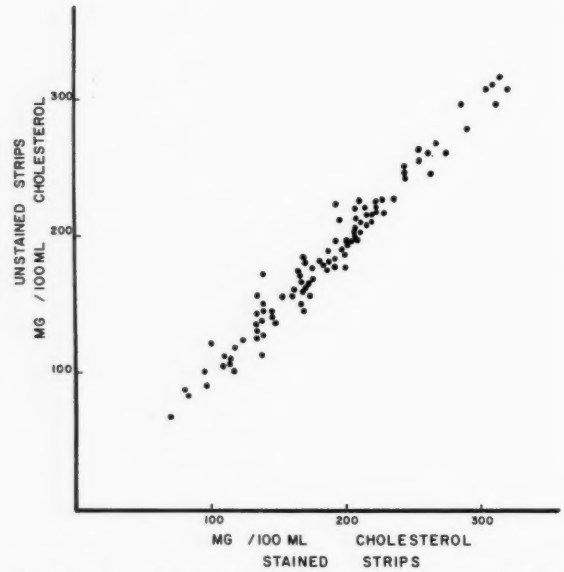

FIG. 3. Comparison of β-lipoprotein cholesterol values obtained from protoporphyrin-stained and unstained electrophoretic strips.

this manner, areas occupied by the alpha- and beta-lipoproteins can be easily identified, marked, and cut from each strip. Cholesterol is extracted in 1 hour by placing each filter paper segment in a test tube containing a measured amount of glacial acetic acid saturated with ferrous sulfate (5). Color is developed from cholesterol by addition of concentrated sulfuric acid to an aliquot of the eluate and estimated on a spectrophotometer at 490 mμ (11).

A series of 100 sera has been subjected to filter paper electrophoresis in order to compare cholesterol recoveries from stained and unstained strips. Lipoprotein cholesterol was isolated directly from one group of strips stained with acid protoporphyrin. Using a duplicate set of electrophoretic separations, the alpha- and beta-lipoprotein areas were identified by a dye marker technique (12), and cholesterol was isolated from unstained strips. No significant differences were observed in the amounts of cholesterol isolated from alpha- or beta-lipoproteins from protoporphyrin-stained or unstained strips (Figs. 2 and 3). These data indicate that lipid-bound protoporphyrin does not significantly interfere with extraction or quantitation of cholesterol by the methods employed. Furthermore, no loss of lipoprotein cholesterol was apparent when strips were treated in the aqueous protoporphyrin system.

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Collecting $C^{14}O_2$ in a warburg flask for subsequent scintillation counting

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» This report describes an improved technique for the collection of $C^{14}O_2$ in a Warburg flask and for the subsequent transfer of the radioactive material for liquid scintillation assay. The CO_2 absorbent used in this work was Hyamine®,[1] which is a high-molecular-weight quaternary amine. Passmann *et al.* were the first to use this substance in collecting $C^{14}O_2$ for liquid scintillation counting, and their article describes some of the important characteristics of Hyamine, for example, its CO_2 absorbing capacity and its effect on counting efficiency (1).

Before we arrived at the method which is subsequently described in detail, we made two unsuccessful attempts to transfer the $C^{14}O_2$ from a Warburg flask to a counting vial. The use of a connecting diffusion tube (9 or 7 mm i.d.) gave poor recoveries (approximately 80% in 3 hours and 90% in 14 hours, after shaking at 37°), and a second procedure, in which the Hyamine was injected into the center well or side arm of the Warburg flask and an aliquot of the solution transferred directly to the vial, was not quantitative because of volume changes during the diffusion period. However, the use of a removable vessel in collecting the CO_2 gave very good recovery and minimized the equipment needed and the possible errors inherent with the other methods. We have used this technique successfully in a preliminary study of the oxidation of C^{14}-labeled fatty acids.

Figure 1 illustrates the arrangement of a Warburg flask for collecting the labeled CO_2. The incubation mixture in the main compartment consists of 5 ml Krebs-Ringer bicarbonate buffer gassed with 95% O_2, 5% CO_2, C^{14}-labeled substrate, and the enzyme system. The side arm contains 0.3 ml 6 N H_2SO_4. The vessel positioned on top of the center well is empty during the enzymatic incubation but, at the termination of the incubation, the vessel serves as a receptacle for 0.5 ml of the hydroxide of Hyamine which is injected from a tuberculin syringe through the rubber cap covering the flask. Once the Hyamine has been injected, the sulfuric acid is poured into the main compartment to release the carbon dioxide from the solution. After allowing the flask to shake in a Dubnoff shaker for 3 hours at 37°, the vessel containing the Hyamine (and carbon-14) is transferred *in toto* to a glass counting vial that contains 5 ml of 0.4% PPO (2,5-diphenyloxazole) and 0.01% POPOP (1,4-bis-2-[5-phenyloxazolyl]-benzene) in toluene. The counting vial is capped, the contents are mixed, and the radioactive content of the solution is measured with a liquid scintillation spectrometer. The presence of any volatile compounds in enzymatic studies might invalidate interpretations based on $C^{14}O_2$ collected in this manner.

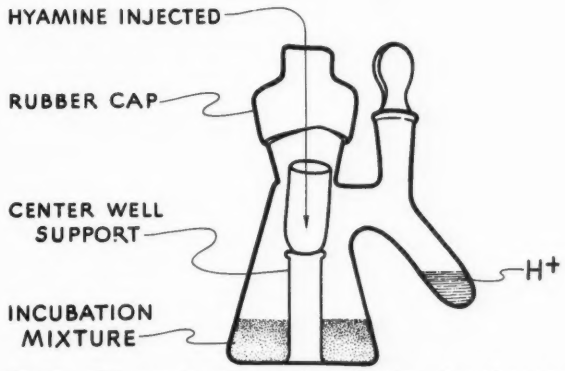

FIG. 1. Components necessary for collection of radioactive carbon dioxide for subsequent liquid scintillation counting.

The recovery of $C^{14}O_2$ from a standard sodium bicarbonate solution was 94.5%, 97.1%, and 98.1%, respectively, at 1, 2, and 3 hours after addition of the acid to the main compartment. The reproducibility of this method can be seen from results obtained with four samples treated in the manner described: 4485, 4441, 4443, and 4439 cpm. The standard deviation is 0.49% of the mean value. The glass vessel, prepared from 10 mm (i.d.) pyrex tubing, did not affect the counting characteristics of carbon-14 in this system. Although Primene (2) was not used in these experiments, it presumably could be used as a less expensive substitute for Hyamine in collecting the carbon dioxide.

* Under contract with the United States Atomic Energy Commission.

[1] Trade name for 1 M *p*-diisobutyl-cresoxyethoxyethyl dimethylbenzylammonium hydroxide in methanol, obtained from Rohm & Hass Co., 712 Locust Street, Philadelphia 5, Pa.

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New Methods

» New Methods is a listing of pertinent references to analytical lipid methods published recently. It is compiled by David Kritchevsky, H. A. I. Newman, and Norman S. Radin. (References opened with No. 1 in the January, 1961, issue of the JOURNAL OF LIPID RESEARCH.)

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The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg
gram	g
milligram	mg
microgram	μg
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m
centimeter	cm
millimeter	mm
millimicron	mμ
square centimeter	cm^2
liter	liter
milliliter	ml
cubic centimeter	cc or cm^3
microliter	μl
counts per minute	cpm
counts per second	cps
millicurie	mc
microcurie	μc
temperature (Centigrade only)	°

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